ISBN 978-0-9861420-0-0

World Clinical *Diabetes*

2015 March 26 First Edition: 1-690

Edited by Silvano Zanuso, PhD, Research Associate

Place of Publication: Pleasanton (CA)

Published by Baishideng Publishing Group Inc

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NAME OF BOOK
World Clinical Diabetes

ISBN 978-0-9861420-0-0

PUBLICATION DATE
March 26, 2015

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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive, Pleasanton, CA 94588-3144, United States
Telephone: +1-925-223-8242
Fax: +1-925-223-8243

E-mail: bpgoffice@wjgnet.com
http: //www.wjgnet.com

World Clinical Hepatology

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

PREFACE

Core progresses in clinical diabetes in 2014

It is a great honour and privilege to introduce to you the latest publications in the field of clinical diabetes. Unlike many specialised journals, *World Clinical Diabetes* (ISBN 978-0-9861420-0-0) intends to bring you a great spread of topics, giving a cross-section of the field and its latest changes and improvements.

It is well known the incidence of non-insulin-dependent diabetes mellitus is increasing worldwide and, though the rise is more marked in developing countries, Europe and North America have not been spared by these "epidemics". The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030, and the total number of people with diabetes is constantly increasing. As a consequence the burden of long-term invalidating complications of diabetes is set to escalate, with an inevitable increase in social and economic costs associated with the disease, especially since diabetic subjects are at higher risk for macro and microvascular disease.

Increased morbidity and mortality from cardiovascular disease have been attributed to the increased incidence of chronic hyperglycemia *per se* and comorbid conditions in diabetic patients as opposed to nondiabetic subjects. These conditions suggest the existence of a pathophysiological category known as "metabolic syndrome".

The metabolic syndrome, which is a cluster of different metabolic risk factors appears to directly promote the development of atherosclerotic cardiovascular disease and to increase the risk of developing type 2 diabetes mellitus.

The metabolic risk factors consist of atherogenic dyslipidemia, elevated blood pressure and elevated plasma glucose, and individuals diagnosed as possessing these metabolic risk factors typically manifest a prothrombotic and a proinflammatory state.

Those underlying conditions are also common to gestational diabetes that, similarly to non-insulin-dependent diabetes mellitus is showing and increase worldwide.

The increasing burden of obesity worldwide seems to be behind the increasing prevalence of those conditions.

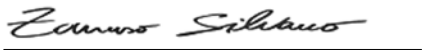
Thus, how can we fight all the negative effects of non-insulin-dependent diabetes mellitus, gestational diabetes and, to a given extent, type 1 diabetes? The answer is quite simple and well known by the clinicians: in fact, since the statement of Joslin in 1959, there is international consensus that the cornerstones of the treatment of diabetes are diet, physical activity and medications; each of those pillars are constantly improved thanks to effort of the clinical and scientific community. Thanks to this multifactorial approach, now we have several possibilities to cure, and even prevent, both type 1 and type 2 diabetes. Previously, research toward a definitive cure was focused on transplantation of the islet cells or parts of the pancreas, now research is focusing on ways to understand this immune attack to find safe ways to block it. Much attention is also aimed at the causes of type 2 diabetes, the main recent theory involves inflammation. In addition, diabetes investigators are working on understanding how islet cells malfunction in type 2 diabetes and what is the genetic basis for this?

Also the "pharmacological side" is very active, and some novel categories of drugs have been proposed in the recent years, like for example the antihyperglycemic therapy based on modulation of the incretin system that has recently emerged.

A vast amount of literature continues to be published also on the effect of exercise on type 2 diabetes. Major developments include advances in basic science that have increased awareness into the effects of exercise on glucoregulation, large clinical trial demonstrating that lifestyle intervention reduces the incidence of type 2 diabetes, meta-analyses of structured exercise intervention in type 2 diabetes showing the effectiveness of exercise in reducing hemoglobin A1c (HbA1c) independently of body weight and the association between exercise training intensity and change in HbA1c. In addition there have been extensive cohort studies demonstrating that low aerobic fitness and low physical activity levels can predict increased risk of overall and cardiovascular disease mortality in people with diabetes, clinical trials verifying the effectiveness of resistance training for improving glycemic control in type 2 diabetes and finally new data on safety of resistance

training in populations at high risk for cardiovascular disease.

The *World Journal of Diabetes*, deeply committed in spreading worldwide the most recent scientific advances in the field of diabetes, features, in this special issue of the journal a vast collection of studies: on type 1 diabetes, type 2 diabetes, gestational diabetes, diabetes complication from different perspectives (nutrition, medications, physical activity, basic science); according to the comprehensive and broad approach which is the distinctive features of the journal.



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March 26, 2015

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (1): Insulin

Insulin and bone: Recent developments

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Received: November 24, 2013 Revised: December 4, 2013
Accepted: December 17, 2013
Published online: February 15, 2014

Abstract

While insulin-like growth factor I is a well-known anabolic agent in bone evidence is beginning to accumulate that its homologue, insulin, also has some anabolic properties for bone. There is specific evidence that insulin may work to stimulate osteoblast differentiation, which in turn would enhance production of osteocalcin, the osteoblast-produced peptide that can stimulate pancreatic β cell proliferation and skeletal muscle insulin sensitivity. It is uncertain whether insulin stimulates bone directly or indirectly by increasing muscle work and therefore skeletal loading. We raise the question of the sequence of events that occurs with insulin resistance, such as type 2 diabetes. Evidence to date suggests that these patients have lower serum concentrations of osteocalcin, perhaps reduced skeletal loading, and reduced bone strength as evidenced by microindentation studies.

Key words: Type 2 diabetes; Insulin; Bone; Osteoblasts; Insulin resistance

Core tip: This is a review of recent publications that suggest an anabolic loop among bone, pancreas, and skeletal muscle.

Original sources: Klein GL. Insulin and bone: Recent developments. *World J Diabetes* 2014; 5(1): 14-16 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i1/14.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i1.14

INTRODUCTION

The interactions between insulin and bone would on the surface appear to be an unlikely subject for an article, let alone a review article, but with the advent of the knockout mouse model many relationships that would not have been obvious now require investigation. The aim of this paper is to provide evidence supporting an anabolic loop including the pancreas, skeletal muscle, and bone.

GROWTH FACTOR

We do not want to confound the anabolic effects of insulin with those of insulin-like growth factor (IGF)-1, although the homology of molecular structure of both molecules may in fact account for some of the anabolic effects of insulin on bone. It should be emphasized at this point that insulin is synthesized in the pancreatic β cells while endocrine IGF-1 is synthesized in the liver. The stimuli for insulin production include glucose and, as we will see, osteocalcin, while endocrine IGF-1 is synthesized by liver in response to growth hormone and the paracrine IGF-1 produced by bone cells, including preosteoblasts and osteoblasts, osteocytes and osteoclasts[1,2] is synthesized in response to stimuli that have not yet been clarified.

While there are copious reports of the anabolic effects of IGF-1 on bone there is a growing amount of data suggesting that insulin itself has an anabolic effect on bone. Suggestions of this effect came from studies involving burned children in which a hyperinsulinemic, euglycemic clamp was employed resulting in an increase in both lean body mass, often indicative of muscle mass,

and bone mass at time of hospital discharge compared to controls, usually between 6 wk to 3 mo post-burn[3]. Moreover, both pre-osteoblasts and osteoblasts manifest different isoforms of the insulin receptor (IR), with IRA being expressed in pre-osteoblasts and IRB being expressed in mature osteoblasts[4]. This specificity suggests that insulin is a critical element in osteoblast differentiation from marrow stromal cells. This may have significance in the generation of the osteoblast peptide osteocalcin, which, as we shall see, has major implications for glucose metabolism. Whether the direct effect of insulin on osteoblasts has clinical significance, however, is not entirely clear. This is in part because the abovementioned report on hyperinsulinemia demonstrated increases in both lean body mass and bone mass[3].

INSULIN

The other side of this proposed loop is the effect of bone on insulin. The stimulus for the work that produced these findings is the knockout mouse model. In this model a significant contribution has been made by Wei *et al*[5] who have most recently reported that osteocalcin stimulates β cell replication in the pancreas *via* a cyclin D1-dependent mechanism utilizing the G-protein coupled receptor family C group 6 member A receptor expressed by these cells. This stimulation occurs during both peak β cell proliferation, which occurs in the perinatal period and in adult mice[5]. Moreover, they described the effects of daily osteocalcin injections in obese type 2 diabetic mice reporting an increase in the number of mitochondria in skeletal muscle as well as an increase in energy expenditure[6], indicating that osteocalcin can also increase muscle work by increasing insulin sensitivity.

Thus these recent data would suggest that under normal conditions insulin may stimulate osteoblast differentiation in order to produce more osteocalcin, which would then stimulate more insulin production by the pancreas and greater insulin sensitivity of skeletal muscle. There are also some recent clinical correlates of these studies in adults. In a recent study Díaz-López *et al*[7] performed a case-control study of 153 diabetic subjects and 306 individually matched controls and found that both the carboxylated and undercarboxylated forms of osteocalcin were lower than matched controls and that carboxylated osteocalcin concentrations were inversely associated with a model assessment of insulin resistance and fasting glucose concentrations. Another report by Gower *et al*[8] indicated that in obese individuals total osteocalcin was directly associated with skeletal muscle but not hepatic insulin sensitivity while undercarboxylated osteocalcin was associated with β cell function in those with abnormal fasting glucose concentrations.

BONE

A major unanswered question is exactly what happens to bone in cases of peripheral insulin resistance? Are the IRs in pre-osteoblasts and osteoblasts down-regulated? We know that osteocalcin levels are lower in type 2 diabetics[7,8]. In addition, we know that insulin resistance is also caused by factors that cause bone resorption, such as the interleukin-6-mediated chronic low grade inflammation that contributes to non-alcoholic fatty liver disease (NAFLD)[9] and excessive glucocorticoid production, another significant contributor to NAFLD[10]. However, we do not at this point know precisely how peripheral insulin resistance affects bone. One conjecture would be that if muscles expend less energy due to their inability to take up glucose then muscle strength may be reduced and skeletal loading may also be consequently decreased. This scenario could explain abnormalities in bone with type 2 diabetes. Were this to be so then bone loss would result in reduced production of osteocalcin and a perpetuation of the problem of peripheral insulin resistance.

So, why has bone loss with type 2 diabetes been so difficult to determine up to now? As summarized by Ferrari[11] in a review article on diabetes and osteoporosis, bone mineral density (BMD) may not be reduced in this condition inasmuch as weight and fat mass must be factored into the BMD determinations. The probability of fracture as assessed by use of the on-line FRAX tool developed by the World Health Organization may also underestimate fracture risk in this condition. As evidence that this may indeed be the case a recent report by Hothersall *et al*[12] examined the files of all hip fractures in Scotland from 2005-2007 and the prevalence of both type 1 and type 2 diabetes in this population. While there was a significant correlation between hip fractures and type 1 diabetes, in which insulin deficiency is the issue, there was no overall increased risk of hip fracture in type 2 diabetes, according to this review. The investigators do, however, state that these findings do not rule out increased risk in sub-groups of type 2 diabetics. While we have demonstrated that osteocalcin, also a marker of bone formation, is lower in patients with type 2 diabetes, not all markers of bone formation or resorption are consistent. For example, Chen *et al*[13] found that while osteocalcin was lower in diabetics *vs* controls, there was no difference in bone specific alkaline phosphatase. Similarly while Bhattoa *et al*[14] found that urinary cross-laps, a resorption marker, was lower in type 2 diabetics *vs* controls while Chen *et al*[13] found that urinary hydroxyproline was elevated.

A new development, however, has shed some light on this problem. In a study that has been Epublished ahead of print, Farr *et al*[15] have reported the use of *in vivo* microindentation of the tibia as an index of bone strength. In this study of 60 post-menopausal women, half of whom had type 2 diabetes, this technique demonstrated decreased bone strength in the diabetic women.

Much more work needs to be done to follow up on these findings but clearly the greater chance of microcracks in the bones of insulin-resistant diabetics may not be detected by bone density determinations.

Therefore, for those who care for diabetic patients, the complications involving bone have been subtle and difficult to detect but as more attention is being paid to

this area the pathogenesis of the bone problem should be more clearly elucidated and new therapeutic targets identified.

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P- Reviewers: Charoenphandhu N, Tarantino G, Traub M
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Hepatitis C virus infection and insulin resistance

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Received: November 9, 2013 Revised: December 20, 2013
Accepted: January 13, 2014
Published online: February 15, 2014

Abstract

Approximately 170 million people worldwide are chronically infected with hepatitis C virus (HCV). Chronic HCV infection is the leading cause for the development of liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and is the primary cause for liver transplantation in the western world. Insulin resistance is one of the pathological features in patients with HCV infection and often leads to development of type Ⅱ diabetes. Insulin resistance plays an important role in the development of various complications associated with HCV infection. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, HCC and resistance to anti-viral treatment. Thus, HCV associated insulin resistance is a therapeutic target at any stage of HCV infection. HCV modulates normal cellular gene expression and interferes with the insulin signaling pathway. Various mechanisms have been proposed in regard to HCV mediated insulin resistance, involving up regulation of inflammatory cytokines, like tumor necrosis factor-α, phosphorylation of insulin-receptor substrate-1, Akt, up-regulation of gluconeogenic genes like glucose 6 phosphatase, phosphoenolpyruvate carboxykinase 2, and accumulation of lipid droplets. In this review, we summarize the available information on how HCV infection interferes with insulin signaling pathways resulting in insulin resistance.

Key words: Hepatitis C virus; Insulin resistance; Insulin receptor substrate 1; Protein kinase B; mammalian target of rapamycin/S6K1; Suppressor of cytokine signaling 3; Glucose transporter-4; Lipid metabolism; Anti-viral therapy

Core tip: Insulin resistance is one of the pathological features in patients with hepatitis C virus (HCV) infection and often leads to development of type Ⅱ diabetes. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, hepatocellular carcinoma and resistance to anti-viral treatment. In this review, we summarize the available information on how HCV infection interferes with insulin signaling pathways.

Original sources: Bose SK, Ray R. Hepatitis C virus infection and insulin resistance. *World J Diabetes* 2014; 5(1): 52-58 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i1/52.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i1.52

INTRODUCTION

Hepatitis C virus (HCV) contains a positive sense single stranded RNA genome and belongs to the family Flaviviridae and genus Hepacivirus[1]. HCV genome, 9.6 kb in length, is composed of a 5' non-translated region (NTR), a long open reading frame (ORF) encoding a polyprotein and a 3' NTR. The ORF encodes a polyprotein of about 3000 amino acids that is translated *via* an internal ribosome entry site at the 5' NTR. The polyprotein is then cleaved by both cellular and viral proteases into at least 10 different proteins[1]. These include three structural

proteins namely, core and two envelope glycoproteins (E1 and E2). In addition, a protein called F or ARFP can be produced from a frame-shift of the core protein[2]. An ion channel protein p7 is formed by cleavage of E2[3]. Non structural proteins of HCV include NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The primary host cell for HCV is hepatocytes but replication may also occur in other cell types, such as peripheral blood mononuclear cells, as well as in B and T cell lines[4,5]. HCV is a major cause of acute and chronic liver disease worldwide. More than 170 million people are currently infected with HCV[6]. Currently HCV vaccine is not available. Acute infection is usually asymptomatic, making early diagnosis difficult. Approximately 70% of acutely infected individuals fail to clear the virus and become chronically infected[7]. Chronic HCV infection is the leading cause for the development of liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and is the primary cause for liver transplantation in the western world. The sustained antiviral response rate in treatment of chronic HCV infection with interferon (IFN)-α with ribavirin is limited (about 30%-40%)[8,9]. Boceprevir and telaprevir protease inhibitors, have been shown to exhibit significantly higher rates of sustained virologic response (SVR) against HCV genotype 1 (about 65%-75%) as compared with peginterferon-ribavirin alone[10,11]. However, use of these antiviral agents display higher incidence of adverse events, such as rash, gastrointestinal disorders, and anemia.

Insulin resistance plays an important role in the development of various complications associated with HCV infection. Recent evidence indicates that HCV associated insulin resistance may result in hepatic fibrosis, steatosis, HCC and resistance to anti-viral treatment[12]. Thus, HCV associated insulin resistance is a therapeutic target at any stage of HCV infection. HCV modulates normal cellular gene expression and interferes with the insulin signaling pathway. The aim of this review is to summarize the currently available information on how chronic HCV infection interferes with insulin signaling pathways resulting in insulin resistance.

GLUCOSE UPTAKE AND INSULIN RESISTANCE

Glucose is a key metabolite essential for the production of energy (mostly ATP) which is required by cells. There are several mechanisms underlying increased glucose production. These include production of free glucose by increased glycogenolysis in the liver, increased gluconeogenesis, activation of forkhead box transcription factor (FoxO1) and improper insulin-glucagon hormonal balance, which stimulates increased glucose production[13]. Several factors contribute to elevated gluconeogenesis in diabetes, namely (1) increased supply of glucogenic precursors to the liver (glycerol, amino acids, free fatty acids), (2) increased lipid content, (3) increased cytokines and adipokines, and (4) decreased insulin receptor (IR) signaling in hepatocytes[13]. Glucose uptake into cells is regulated by the action of specific hormones, namely insulin and glucagon. Insulin is a peptide hormone secreted by the β-cells of the pancreatic islets of langerhans and maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects[14]. The ability of insulin to stimulate glucose uptake into tissues is central to the maintenance of whole-body glucose homeostasis[15]. Type Ⅱ diabetes mellitus (T2DM), occurs when the production of insulin is not sufficient to overcome a difficulty the body has in properly using insulin. This difficulty is called insulin resistance, resulting in increased glucose levels. Both forms of diabetes can pose an increased risk of major lifelong complications. In the case of insulin resistance, this includes a fivefold increased risk of coronary vascular disease, diabetic retinopathy and neuropathy[16-19]. Fatty liver is relatively common in overweight and obese persons with T2DM and is an aspect of body composition related to severity of insulin resistance, dyslipidemia, and inflammatory markers[20].

Glucose transporter-4 (GLUT-4) was shown to be the major isoform responsible for enhanced glucose uptake into muscle and adipose tissues following the secretion of insulin into the bloodstream[21,22]. The process of glucose uptake by cells requires a series of events to take place in a timely manner. It involves the binding of insulin to the IR resulting in subsequent phosphorylation and activation of IR substrate 1 and 2 (IRS-1/IRS-2), central molecules of the insulin signaling cascade[23,24]. This in turn activates protein kinase B (AKT) by phosphorylation of Ser^{473} and Thr^{308} residues. Activated AKT causes the translocation of GLUT-4 from intracellular compartments to the cell surface where it is required for glucose uptake[25]. Any change in the signaling is likely to induce insulin resistance which is associated with a number of pathophysiological changes including glucose intolerance, obesity, dyslipidemia and hypertension. Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone insulin. The body produces insulin, but the cells in the body become resistant to insulin and are unable to use it as effectively, resulting in an attenuated biological response, leading to hyperglycemia[26]. Accumulation of ectopic lipid metabolites, activation of the unfolded protein response pathway, and innate immune pathways have all been implicated in the pathogenesis of insulin resistance[27]. During the course of insulin resistance several inflammatory cytokines and lipid metabolites, like free fatty acids, interrupt with the normal insulin signaling and promote T2DM.

CHRONIC HCV INFECTION AND INSULIN RESISTANCE

Epidemiological studies suggest that patients with chronic HCV infection have a significantly increased preva-

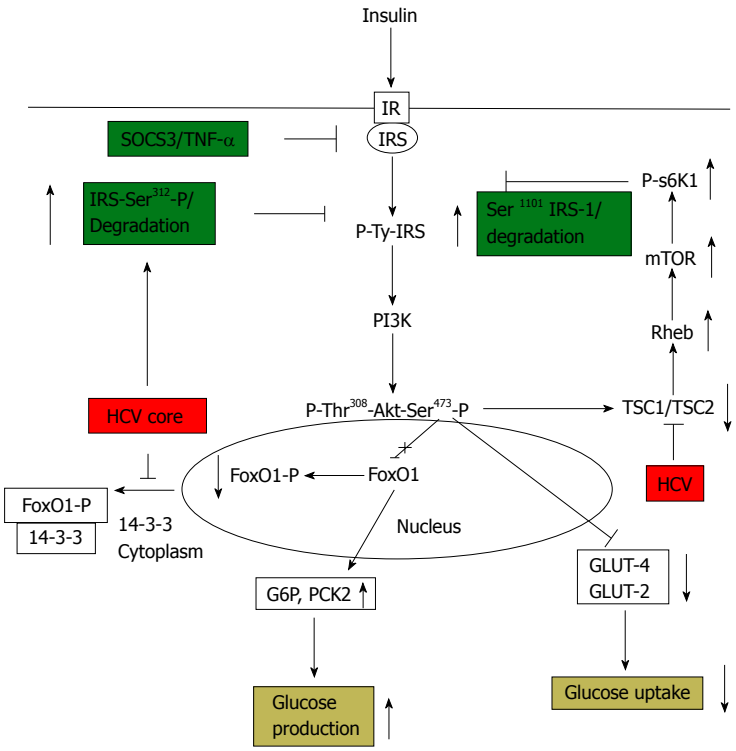

Figure 1 Schematic showing the interference of Hepatitis C virus in the insulin signaling pathway. Hepatitis C virus (HCV) core protein is known to up regulate Ser^{312} phosphorylation of insulin receptor substrate (IRS)-1 leading to degradation of IRS-1, the key molecule involved in propagation of insulin signal downstream from the insulin receptor (IR). HCV infection is also known to down regulate TSC1/TSC2 complex, resulting in subsequent upregulation of mTOR/S6K1 which leads to Ser^{1101} phosphorylation of IRS-1 and its subsequent degradation. A role of HCV mediated upregulation of SOCS3 and tumor necrosis factor-α (TNF-α) has also been proposed which leads to degradation and blocking of IRS-1 function. HCV also upregulates glucose 6 phosphatase (G6P), phosphoenolpyruvate carboxykinase 2 (PCK2) leading to increased glucose production, and down regulates glucose transporter (GLUT)-4, GLUT-2, leading to decreased glucose uptake by hepatocytes. Overall, these alterations lead to insulin resistance. mTOR: Mammalian target of rapamycin.

lence of T2DM as compared to hepatitis B virus infected patients[28-30]. Both insulin resistance and diabetes can adversely affect the course of chronic hepatitis C (CHC), leading to enhanced steatohepatitis and liver fibrosis[30-32]. Insulin resistance, associated with type 2 diabetes, can promote fatty liver, and excessive hepatic accumulation of fat may promote insulin resistance and therefore contribute to the pathogenesis of the metabolic syndrome[33]. Insulin resistance is a critical component of type 2 diabetes mellitus pathogenesis. Several mechanisms are likely to be involved in the pathogenesis of HCV-related insulin resistance[34]. Several cellular lesions have been associated with insulin resistance, but the precise mechanism by which HCV induces insulin resistance remains elusive with numerous viewpoints and opinions[30].

Impairment of IRS-1 and IRS-2 expression has been observed in the liver of patients with chronic HCV infection, as well as in HCV core transgenic mice, and from *in vitro* cell culture system[35-38]. HCV mediates dysfunction of the insulin signaling pathways *via* several distinct mechanisms, such as upregulating the expression of suppressors of cytokine signaling 3 expression[35], down regulation of peroxisome proliferator-activated receptors gamma (PPARγ)[36], activation of mammalian target of rapamycin (mTOR)/S6K1 pathway[38], and increased tumor necrosis factor-α (TNF-α) secretion[39].

MODULATION OF IR SUBSTRATE BY HCV

HCV modulates insulin signaling and IRS-1 *via* multiple mechanisms which have been presented in Figure 1. Ser/Thr phosphorylation of IRS-1 inhibits its association with the IR, which in turn inhibits tyrosine phosphorylation of IRS-1, required for its activation, and promotes degradation. Upregulation of serine phosphorylation of IRS-1 is a key negative feedback mechanism under physiological conditions to prevent the action of insulin. In an insulin-resistant state, an imbalance occurs between positive IRS-1 Tyr-phosphorylation and negative Ser-phosphorylation of IRS-1[40]. HCV core protein expression in hepatocytes upregulates Ser^{312} phosphorylation status of IRS-1 and modulates downstream Akt activity by inhibiting Thr^{308} phosphorylation[37]. Ser^{312} and Ser^{1101} phosphorylation of IRS-1 inhibits its association with the IR and stimulates degradation. HCV core protein induces insulin resistance by increasing Ser^{312} and Ser^{1101} phosphorylation, marking its for degradation *via* the activated mTOR/S6K1 pathway[38], and subsequently blocking Tyr-phosphorylation of IRS-1 and Thr^{308} phosphorylation of Akt for the inhibition of glucose uptake. Activation of mTOR signaling also plays a key role in modulating IRS-1 activity. HCV genotype 2a infection significantly down-regulates the expression of TSC1/TSC2, which in turn results in activation of downstream mTOR and S6K1[38]. Phosphorylation of IRS-1 at Ser^{1101} *via* the mTOR-S6K1 pathway may release IRS-1 from intracellular complexes, thereby enabling its degradation[41]. HCV significantly increases Ser^{1101} phosphorylation of IRS-1, which enables its degradation[38].

A decrease in expression of IRS-1 and IRS-2, in patients with HCV infection has also been reported[35]. Down-regulation of IRS-1 and IRS-2 was also seen in HCV core-transgenic mice livers and HCV core-transfected human hepatoma cells[35]. HCV core up-regulated suppressor of cytokine signaling 3 (SOCS3) and caused ubiquitination of IRS-1 and IRS-2. HCV core-induced down-regulation of IRS-1 and IRS-2 was not seen in

SOCS3(-/-) mouse embryonic fibroblast cells, indicating the important role played by SOCS3 in mediating down regulation of IRS-1[35]. There have been reports that HCV genotypes might play an important role in deciding the pathway by which it impairs insulin signaling. It has been shown that the core protein of HCV genotype 3a promoted IRS-1 degradation through the downregulation of PPARγ and by upregulating the SOCS7, the core protein of genotype 1b activated the mTOR[36].

TNF-α, released in an excess may promote phosphorylation of serine residues of IRS-1 eventually leading to the downregulation of downstream insulin signaling molecule Akt. HCV core protein increases the expression level of TNF-α and promotes insulin resistance[42].

IMPAIRED LIPID AND GLUCOSE METABOLISM BY HCV

Insulin resistance is strongly influenced by abnormalities in lipid metabolism. Any dysfunction of the lipid metabolism triggers lipotoxicity through the production of free fatty acids thereby promoting insulin resistance[43]. HCV core protein down-regulates microsomal triglyceride transfer protein, an enzyme that mediates lipid translocation to the endoplasmic reticulum membrane and decreases the assembly of very low density lipoproteins[44]. It has been observed that HCV promotes fatty acid synthesis by the upregulation of lipogenic gene sterol regulatory element binding protein 1c which promotes the transcriptional activation of other lipogenic genes like acetyl CoA carboxylase, ATP citrate lyase, hydroxymethylglutaryl CoA reductase[45].

HCV infection promotes the expression of gluconeogenic genes namely, glucose 6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase 2 (PCK2) resulting in increased glucose production and enhanced insulin resistance[46,38]. HCV also down regulates the expression of GLUT4, which is necessary for uptake of glucose. This results in a decreased glucose uptake and increased plasma glucose, leading to development of insulin resistance[38].

A schematic showing how HCV interferes with insulin signaling pathway, leading to insulin resistance is presented in (Figure 1). HCV modulates functioning of IRS-1 *via* multiple mechanisms, including up regulation of Ser^{312} or Ser^{1101} phosphorylation which leads to degradation of IRS-1. HCV also upregulates SOCS3 and down regulates TSC1/TSC2 leading to blocking of insulin signaling. HCV infection leads to increased gluconeogenesis *via* up regulation of G6P and PCK2. GLUT-4, and GLUT-2 expression is also down regulated by HCV leading to decreased glucose uptake. Overall, all these alterations by HCV leads to development of insulin resistance.

INSULIN RESISTANCE AND LIVER DISEASE PROGRESSION

The metabolic syndrome is a constellation of problems that includes insulin resistance, obesity, hypertension, and hyperlipidemia[47]. Increasingly, components of the metabolic syndrome are being linked to various forms of cancer, including the risk of developing HCC. IR is induced by HCV-4 irrespective of severity of liver disease. IR starts early in infection and facilitates progression of hepatic fibrosis and HCC development[47]. HCC patients showed higher IR frequency, and moderate to high viral load associated with high HOMA-IR in CHC and HCC[47]. Insulin resistance associates with a higher risk of HCC in cirrhotic HIV/HCV-co-infected patients also[48]. There are many causes of HCC, and nonalcoholic fatty liver disease (NASH) is emerging as a leading risk factor owing to the epidemic of obesity and T2DM. The mechanisms leading to HCC in obesity and T2DM likely involve interactions between several signaling pathways, many of which are modulated by HCV infection, and also include oxidative stress, inflammation, oncogenes, adiponectins, and insulin resistance associated with visceral adiposity and diabetes[49].

Insulin resistance and subsequent hyperinsulinemia are highly associated with fatty liver disease and is an important risk factor for the progression of fibrosis in CHC[50,51]. From metabolic aspect, HCV infection resembles NASH in numerous features, such as the presence of steatosis, serum dyslipidemia, and oxidative stress in the liver[52]. On the other hand, there are noticeable differences between hepatitis C and NASH, in the fact that HCV modulates cellular gene expression and intracellular signal transduction pathways, while such details have not been noted for NASH. HCV core protein expression leads to the development of progressive hepatic steatosis and HCC in transgenic mice[53]. Hepatic steatosis is known to occur at a high rate (40%-86%) in chronic HCV patients, and a close relationship between steatosis and intrahepatic core protein expression has been noted[54]. Insulin resistance is a prominent mechanism linking steatosis and fibrogenesis although this link is complex and not properly understood.

CLINICAL IMPLICATIONS OF HCV-MEDIATED INSULIN RESISTANCE

Several epidemiological, clinical and experimental data show that HCV plays a direct role in perturbing glucose metabolism, leading to both insulin resistance and diabetes[28-30]. Curing HCV results in the amelioration of insulin resistance and decreased incidence of diabetes after the end of therapy[55,56]. In the only trial that used the antidiabetic metformin[57], only a marginal, nonsignificant increase of the SVR rate was observed, despite an increased virological response after 4 wk of triple therapy. The data reported in a study using different schedules containing the antiglycaemic PPAR-γ agonist pioglitazone[58] are discouraging. Overall, the administration of insulin sensitizers together with the standard of care has not only failed to improve the virological response to therapy, but has also fallen short of providing much useful insight into the mechanisms linking reduced response

to insulin resistance[59]. Early sulfonylureas although useful in lowering blood glucose level, were associated with significant off-target effects, and the biguanide phenformin was discontinued due to adverse events[60]. Although metformin is in the same drug class, it has a better safety profile and is now recommended as first-line treatment of diabetes during HCV infection.

THERAPEUTIC APPROACHES AND FUTURE GOALS

Treatment for HCV induced insulin resistance is highly linked with anti-viral treatment. Treatment of chronic HCV infection has 2 goals. The first is to achieve SVR (*i.e.*, sustained eradication of HCV, which is defined as the persistent absence of HCV RNA in serum 6 mo or more after completing antiviral treatment). The second goal is to prevent progression to cirrhosis, HCC, and decompensated liver disease requiring liver transplantation. The treatment of HCV has evolved over the years. Current treatment options include combination therapy consisting of ribavirin and pegylated IFN. Protease inhibitors are emerging as a third feature of combination therapy. The sustained antiviral response rate in treatment of chronic HCV infection with IFN-α and ribavirin is limited (about 30%-40%)[8,9]. Boceprevir and telaprevir protease inhibitors have been shown to exhibit significantly higher rates of SVR against HCV genotype 1 (65%-75%) as compared with peginterferon-ribavirin alone[10,11]. More recently, sofosbuvir has also been used for treatment along with ribavirin, with significant increased SVR[61]. However, use of these antiviral agents display higher incidence of adverse events, such as rash, gastrointestinal disorders, and anemia. Thus, development of therapies with less side effects is desirable.

The prevalence of HCV antibodies in the type 2 diabetic population ranges between 1.78% and 12.1%[62]. Several cross-sectional studies have found a higher prevalence of HCV antibodies in type 2 diabetic patients than expected in the general population[62,63]. Early phase and total insulin secretion are determined using oral glucose tolerance testing (OGTT), Insulin sensitivity was measured directly by steady-state plasma glucose concentration during insulin suppression test. Fasting plasma glucose $\geqslant$ 126 mg/dL or 2-h plasma glucose > 200 mg/dL during OGTT are generally used as criteria for diagnosis of diabetes[64]. Well controlled DM was defined when the HbA1c level was < 7%. Agents used in diabetic therapy include the following: sulfonylureas, biguanides, alpha-glucosidase inhibitors, thiazolidinediones, Meglitinide derivatives *etc*[60]. Although effective in reducing blood glucose levels, early sulfonylureas were associated with significant off-target effects, and the biguanide phenformin was discontinued due to adverse events[60]. Although metformin is in the same drug class, it has a better safety profile and is now recommended as first-line treatment. However, many patients require additional glucose control treatment with an agent that has a complementary mechanism of action like metformin. Some common drugs used for treatment of T2DM available in the market include metformin oral, actos oral, Byetta subQ, Janu*via* oral, *etc*.

Another possible way of reversing insulin resistance would be *via* targeting the signaling components in the insulin signaling pathway modulated by HCV. For instance, we have shown that HCV up regulates phospho-S6K1, which stimulates degradation of IRS-1[38]. Thus, targeting phospho-S6K1 would be a target against HCV induced insulin resistance. These studies have not been done yet, so at this time it will be difficult to comment on the predictive outcome on reversal of insulin resistance. Use of specific inhibitors of SOCS-3, which may become useful to correct resistance to both insulin and IFN-α, are not available for clinical use. Alternatively, one may envision inhibiting TNF-α by administering infliximab or similar agents. IR also results from uncontrolled diet and life style. Regulation of weight, diet, and life style management will also be key in managing IR.

ACKNOWLEDGMENTS

We thank and Lin Cowick for preparation of the manuscript.

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P- Reviewers: Efanov AM, Teeter JG, Traub M, Vestergaard ET
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
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ISBN 978-0-9861420-0-0

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New insights into insulin: The anti-inflammatory effect and its clinical relevance

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Author contributions: Sun Q drafted the manuscript; Gao F contributed to the review and approved the final version of the manuscript; Li J revised and edited the manuscript.
Supported by The State Key Program of National Natural Science Foundation of China, No. 81030005; The National High Technology Research and Development Program of China, No. 2014AA020542; and National Natural Science Foundation of China, No. 31371150
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Received: November 28, 2013 Revised: February 8, 2014
Accepted: March 3, 2014
Published online: April 15, 2014

Abstract

Hyperglycemia, a commonly exhibited metabolic disorder in critically ill patients, activates the body's inflammatory defense mechanism, causing the waterfall release of numerous inflammatory mediators and cytokines, and eventually leads to organ damage. As the only glucose-lowering hormone in the body, insulin not only alleviates the detrimental effects of hyperglycemia through its metabolic regulation, but also directly modulates inflammatory mediators and acts upon immune cells to enhance immunocompetence. In this sense, hyperglycemia is pro-inflammatory whereas insulin is anti-inflammatory. Therefore, during the past 50 years, insulin has not only been used in the treatment of diabetes, but has also been put into practical use in dealing with cardiovascular diseases and critical illnesses. This review summarizes the recent advances regarding the anti-inflammatory effects of insulin in both basic research and clinical trials, with the hope of aiding in the design of further experimental research and promoting effective insulin administration in clinical practice.

Key words: Insulin; Inflammation; Hyperglycemia

Core tip: Hyperglycemia is closely correlated with poor outcomes of morbidity and mortality in critically ill patients. As the only glucose-lowering hormone in the body, insulin not only alleviates the detrimental effects of hyperglycemia through its metabolic regulation, but also directly modulates inflammatory mediators and acts upon immune cells to enhance immunocompetence. This review summarizes the recent advances regarding the anti-inflammatory effects of insulin from our laboratory as well as others, in the hope of leading to a better understanding of this old, classic and wonder hormone and its wider and effective applications in clinical practice.

Original sources: Sun Q, Li J, Gao F. New insights into insulin: The anti-inflammatory effect and its clinical relevance. *World J Diabetes* 2014; 5(2): 89-96 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/89.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.89

INTRODUCTION

Since its discovery in 1921, the importance of insulin in glucose homeostasis has been established, and it is universally used as a therapeutic agent for diabetes mellitus. Thousands of lives have been saved and many scientists were drawn into the study of this wonder drug. Under continuous intensive research, the mechanisms underlying the effect of insulin in its metabolic modulation, mainly glucose homeostasis, has become clearer, but

there remains much interest in the elucidation of further effects of insulin.

Glucose-insulin-potassium (GIK) has been used as an adjunctive therapy in patients with acute myocardial infarction (AMI) since its introduction in 1962. However, the mechanism underlying GIK's cardioprotection has remained largely speculative and controversial during the past 50 years. It was not until early in this century that we provided convincing *in vivo* evidence that insulin, rather than glucose or potassium, is the predominant protective component of GIK, and demonstrated for the first time that insulin exerted anti-apoptotic and pro-survival effects in the ischemic/reperfused (I/R) myocardium through the PI3K-Akt-eNOS-NO signaling pathway[1]. This prompted us to conceive the notion of the "survival signal", a new mechanism of cell protection which is totally independent of the metabolic effects of insulin, and explained its cardioprotective effects. In 2001, the classical landmark clinical trial by van den Berghe[2] revealed that maintaining blood glucose at or below 110 mg/dL with low-dose insulin infusion, significantly reduced mortality and morbidity resulting from multi-organ failure among critically ill patients in the surgical intensive care unit (ICU). A further study reported that markers of inflammation, such as intercellular cell adhesion molecular-1 (ICAM-1) and E-selectin were suppressed in the liver of these patients as was inducible NO synthase (iNOS) expression, which is mainly in monocyte/macrophage cells[3], suggesting an anti-inflammatory role for insulin. This article will summarize the relationship between insulin, glucose and inflammation, and discuss the implications for the management of patients with AMI and critical illness.

GLUCOSE, OXIDATIVE STRESS AND INFLAMMATION

Hyperglycemia is common in critical illness, and may lead to severe complications. It has been reported that pronounced hyperglycemia is associated with poor outcomes of morbidity and mortality in patients with AMI, stroke and coronary artery bypass grafting[4-6]. Glucose is pro-inflammatory, and hyperglycemia is even detrimental to these patients. A total of 75 g glucose intake causes acute oxidative and inflammatory stress, as reflected in increased superoxide radical O_2^- generation by polymorphonuclear leukocytes, mononuclear cells and the enzyme nicotinamide adenine dinucleotide phosphate[7]. Free radical O_2^- generation, on the one hand, reduces NO bioavailability, as it combines with NO to form peroxynitrite $ONOO^-$; on other hand, it activates a number of redox-sensitive major pro-inflammatory transcription factors such as nuclear factor kappa B (NFκB), activator protein-1 (AP-1), hypoxia induced factor-α (HIF-α) and early growth response-1 (Egr-1), leading to increased transcription of the pro-inflammatory genes and thus inflammation[8-10]. Meanwhile, glucose increases the expression of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in mononuclear cells. Moreover, it has led to increased TNF-α and IL-6 concentrations in plasma in a steady state of hyperglycemia with intravenous insulin secretion with somatostatin[11]. To sum up, glucose, oxidative stress and inflammation are inter-related, with reciprocal causation. As the only glucose-lowering hormone in the body, insulin therapy alleviates the detrimental effects of hyperglycemia through metabolic regulation, therefore hyperglycemia is pro-inflammatory whereas insulin is anti-inflammatory.

INSULIN MODULATES INFLAMMATORY MEDIATORS

The discovery of the anti-inflammatory effect of insulin can be traced back to the observation that insulin exerts a vasodilatory effect through endothelial NO release in arteries, veins and capillaries[12,13]. By inducing vasodilatation, it reduces leukocyte adhesion to the endothelium and subsequent infiltration. Furthermore, it has inhibitory effects on platelet adhesion and aggregation.

Studies have further confirmed that insulin suppressed three important inflammatory mediators: intercellular cell adhesion molecular-1 (ICAM-1), MCP-1 expression and NFκB binding in human aortic endothelial cells *in vitro*[14,15]. These suppressive effects can be blocked by the NOS inhibitor N(G)-nitro-L-arginine, indicating the effects are mediated by NO release. Among all the pro-inflammatory cytokines, TNF-α is the most active one in triggering the production of other cytokines such as IL-6 and other expression molecules[16]. We provided direct evidence in myocardial ischemia/reperfusion (I/R) rats that insulin inhibits TNF-α induction locally and systemically, and demonstrated for the first time that *in vitro* treatment with insulin attenuated I/R-induced TNF-α production in cardiomyocytes *via* the Akt-eNOS-NO signaling pathway[17]. Polymorphonuclear neutrophils (PMN) are the first defense line against infection and invasive microorganisms. Adherence of PMN to endothelial cells is an early requisite event in I/R-induced inflammatory injury. Thus we performed *in vivo* and *in vitro* experiments in a rabbit model to investigate whether insulin inhibits PMN-mediated adherence[18]. It was found that insulin reduced P-selectin and ICAM-1 expression in endothelium which mediates the initial interaction between PMNs and the endothelial cell surface, thus insulin attenuated PMN adherence and I/R-induced inflammatory injury. The Akt-eNOS-NO signaling pathway was involved in these effects. Moreover, insulin has been reported to ameliorate the endotoxin-induced systemic inflammatory response by decreasing IL-6, TNF-α expression and increasing the anti-inflammatory cascade in the context of normoglycemia in rat[19] and porcine models[20]. All these data indicate that insulin alleviates inflammation through suppression of pro-inflammatory cytokines and immune mediators,

pointing strongly to its role as an anti-inflammatory agent.

INSULIN SUPPRESSES TOLL-LIKE RECEPTOR EXPRESSION

Toll-like receptors (TLRs) are a variety of conserved pattern recognition receptors that have been implicated in innate immune responses. Accumulating evidence suggests that TRLs play an essential role in tissue inflammation and damage such as cardiac I/R, post-ischemic remodeling and atherosclerosis[21-23]. TLR signaling and its critical roles in inflammatory cardiac conditions has been intensively studied, especially TRL2, TRL4' role with myocardial infarction and reperfusion injury. TRL2 aggravated myocardial tissue injury in I/R-based experimental animal models and its deletion was associated with a smaller MI size compared with control[24]. The TLR-deficient model, $TLR2^{-/-}$ mice, exhibited improved left ventricular dysfunction following I/R[25]. Besides, administration of anti-TLR2 antibody prior to reperfusion reduced MI sites and preserved cardiac function. TLR4 is the specific receptor of endotoxin, thus it mediates inflammatory changes induced by endotoxins. Oyama *et al*[26] first demonstrated that TLR4-deficient mice had more than 50% reduction in MI area, which was associated with attenuated myocardial inflammation, as evidenced by less neutrophil infiltration and fewer lipid peroxides. Inhibited by eritoran, a specific TLR4 antagonist, resulted in a 40% reduction in MI and decrease in TNF-α, IL-1β, IL-6 and MCP-1 expression[27,28]. Moreover, TRL4 has been found to act as a determinant of neutrophil infiltration after global MI through mediating KC and MCP-1 expression[29]. Suppression of TRL signaling is associated with smaller MI size and is beneficial in I/R-based animal models. It has been reported that insulin infusion (2 U/h) with type 2 diabetes (T2D) patients within 2 h has significantly suppressed TLR1, -2, -4, -7 and -9 mRNA expressions in MNCs, and this prompt suppression may be mediated by the suppression of PU.1 binding and subsequent activation of TLRs[30]. Thus, insulin suppresses the expression of several TLRs at the transcriptional level and alleviates TRL-mediated inflammatory injury.

INSULIN ACTS UPON IMMUNE CELLS

Peripheral blood mononuclear cells (PBMCs) is a critical component in the immune system, and mainly comprised of lymphocytes and monocytes. Investigations have been conducted to study the effects of insulin upon mononuclear cells in obese non-diabetic subjects[31]. The results showed that insulin reduced activation of the pro-inflammatory transcription factor NFκB, with downregulation of plasma soluble intercellular adhesion molecular-1, which facilitates the attachment of monocytes to endothelial cells and chemotactic factor MCP-1, which encourages monocyte migration into the subintimal space. This suppressive effect on NFκB in PBMC has also been reported in critically ill patients with intensive insulin therapy[32]. Similarly, Egr-1, another important pro-inflammatory transcription factor, was notably reduced in mononuclear cells with insulin treatment, resulting in decreased plasma concentrations of tissue factor and plasminogen activator inhibitor-1 (PAI-1)[33]. Taken together, insulin suppresses pro-inflammatory transcription factors in mononuclear cells and the subsequent inflammatory mediators regulated by them, thus ameliorating MNC-mediated inflammation.

Monocytes/macrophages (Mo/Mϕ) initiate immune and inflammatory responses. Insulin administration (10^{-7} mmol/L) retarded macrophage apoptosis and enhances Bclxl mRNA expression by activating phosphatidylinositol 3'-kinase (PI3K) in a dose-dependent manner, thus improving macrophage survival[34]. Use of wortmannin, a specific inhibitor of PI-3K, has further confirmed its position in the anti-apoptotic effect of insulin in lipopolysaccharide-challenged THP-1 cells[35]. HLA-DR is a cluster of membrane molecules of Mo/Mϕ which are involved in the Mo antigen presentation to T cells. The intensity of HLA-DR expression is associated with immunocompetency of Mo/Mϕ[36]. Insulin treatment with blood glucose maintained between 4.4-6.1 mmol/L increased HLA-DR expressions of peripheral Mo cells. This upregulation means enhanced antigen presentation of Mo cells, indicative of improved immune function. Moreover, the phagocytosis, chemotaxis, and oxidative burst capacity of Mo have also been assessed in a burn-injured rabbit model, suggesting that insulin improved the capacity for phagocytosis and oxidative burst, but had no effect on chemotaxis[37].

T cell differentiation is important in the immune response. A single naïve T cell under cell differentiation is able to generate multiple subsets of memory T cells with different phenotypic and functional properties in response to infections, resulting in acquisition of immune functions required for pathogen clearance. Insulin was first confirmed to induce a shift in Th cell differentiation toward Th2 cells which is involved in secretion of inflammatory mediators (IL-4, IL-10, IL-13, *etc*.) and enhanced antibody-mediated responses[38]. Myocarditis is a severe disease of myocardial inflammation and often results from an autoimmune reaction. Significant T cell reduction was observed in cardiac myocarditis[39]. Thus, we investigated the effect of insulin on myocardial inflammation in experimental myocarditis in mice and its potential role in T cell regulation. The results showed that insulin promoted T cell recovery, particularly $CD3^+$ T cells without changing the naïve-to-memory T-cell ratio and had a direct effect on T cell proliferation, thus alleviating myocarditis[40]. It is possible that insulin may promote T cell recovery in myocarditis, especially in diabetic or hyperglycemic patients.

ANTI-INFLAMMATORY EFFECTS OF INSULIN IN HUMAN STUDIES

Cardiovascular disease (CVD) is the leading cause

of death worldwide, and remains a great challenge in healthcare. Various risk factors of CVD, including hypertension, diabetes and smoking, can initiate a chronic inflammatory reaction. Accumulating epidemiological and clinical studies have found strong and consistent relationships between markers of inflammation and the risk of future cardiovascular events[41]. Thus, inflammation is established as a definitive cardiovascular risk factor.

Hyperglycemia is pro-inflammatory and damaging, especially in critically ill patients. Pronounced hyperglycemia at hospital admission is associated with poor outcomes of morbidity and mortality in patients with AMI, thus effective glucose management is a necessary therapeutic intervention. It has been shown in large pilot studies, Diabetes and Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI)[42] and the Estudios Cardiologicos Latinoamerica (ECLA) study[43], that small doses of intravenously delivered insulin markedly improved clinical outcomes in patients with AMI. There was a marked 29% reduction in 1-year mortality in the insulin-glucose infusion group in the 1995 DIGAMI study, and a statistically significant reduction in mortality and a consistent trend toward fewer in-hospital events in the GIK group in the 1998 ECLA pilot trial, possibly as a result of rigorous glycemic control. The anti-inflammatory effect of insulin have been applied clinically. Plasma C-reactive protein (CRP) and serum amyloid A (SAA) concentrations are the two accepted markers of systemic inflammation which were impressively reduced to 40% in patients with AMI when treated with low-dose insulin infusion[44]. As the CRP concentration is correlated with the size of the infarct in AMI, a reduction is indicative of insulin's cardioprotective effects. Moreover, intensive insulin therapy has been given to critically ill patients in surgical and medical ICUs with improved outcomes[2,45]. In 1548 critically ill patients undergoing surgery, insulin infusion which maintained fasting blood glucose concentrations under 110 mg/dL dramatically improved the clinical outcomes with a reduction in total mortality by 48%, the incidence of bacteremia by 46%, acute renal failure requiring dialysis by 41%, ICU poly-neuropathy by 44%, and the need for red cell transfusion by 50% when compared with controls[2]. Mortality and morbidity in the surgical ICUs was dramatically reduced, as was morbidity in medical ICUs. No other agent has been shown to reduce mortality and morbidity by this magnitude in so many diverse ways in the ICU setting. Glucose control seems crucial, but several potential mechanisms may add to the benefits, including reduction of systemic inflammation[46], prevention of immune dysfunction[37], and protection of the endothelium[3,47]. The exact mechanisms underlying this simple and cost-effective intervention need further investigations.

INSULIN RESISTANCE AND INFLAMMATION

Insulin resistance (IR) is a pathological condition wherein insulin-stimulated glucose uptake and clearance in targeted organs are decreased. A few studies suggested that obesity, inflammation and IR are inextricably linked through the actions of specific inflammatory immune cells. The development of IR is thought to occur in response to increased production of pro-inflammatory cytokines by adipose tissue in obesity, that then have an inhibitory effect on insulin signaling pathways in multiple tissues. TNF-α was first found to be increased in adipose tissue of obese mice and able to induce IR[48]. In animal studies, administration of exogenous TNF-α induces IR, whereas neutralization of TNF-α improves insulin sensitivity. IL-1β, another key inflammatory cytokine, interferes with insulin signaling which leads to IR. TNF-α, and more generally, inflammation, activates and increases the expression of several proteins that suppress and impair specific pathways of insulin signaling, making the human body less responsive to insulin and increasing the risk of IR. In turn, IR states are pro-inflammatory. Increased levels of markers and mediators of inflammation such as fibrinogen, CRP, IL-6, PAI-1 and white cell count were shown to correlate with T2D[49-53]. These inflammatory mediators perpetuate and promote the progression of IR. Polycystic ovary syndrome, another IR state, was found to have chronic low-grade inflammation[54]. In other words, inflammation causes IR, and IR is inflammatory. Thus, anti-inflammatory treatment could be proposed as a therapeutic strategy in the treatment of IR.

ANTI-INFLAMMATION THERAPY FOR INSULIN RESISTANCE

Inflammation is hallmark of diabetes and a main cause of its long-term complications. Particularly in obese conditions in humans and animals, it contributes to the pathogenesis of T2D through IR. Therefore, anti-inflammation therapy may be proposed as a strategy for the improvement of IR.

TNF-α is a critical mediator of inflammation, and its increased expression was found to be associated with IR in the adipose tissue of obese mice[48]. *In vitro* studies demonstrated that TNF-α had a direct inhibitory effect on insulin signaling and impaired insulin-stimulated glucose uptake and metabolism in human subjects[55]. Clinically, neutralization of TNF-α with infliximab in patients with rheumatoid arthritis has significantly improved IR as reflected by the significant reduction in the Homeostasis Model Assessment Index[56]. Peroxisome proliferator-activated receptors (PPAR)γ inactivation leads to suppression of IRS-2, which is a signaling molecular in insulin pathways, thus further promotes IR. The anti-diabetic thiazolidinediones (TZDs), which include pioglitazone, rosiglitazone and troglitazone, are clinically used to improve insulin sensitivity in patients with T2D by lowering free fatty acids (FFA) in blood by activating PPARγ. Aspirin, another therapeutic agent, inhibits the activity of multiple kinases induced by TNF-α, and thus enhances insulin sensitivity by protecting proteins

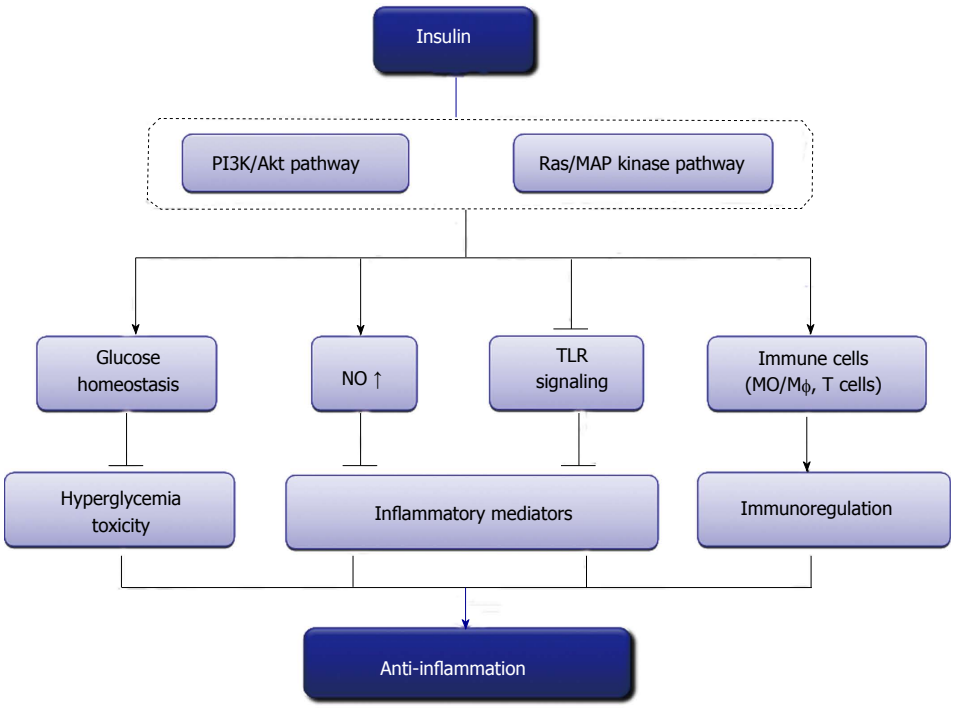

Figure 1 Anti-inflammatory effects of insulin. TLR: Toll-like receptor.

from serine phosphorylation[57]. Statins, as a class of anti-inflammatory drugs, have been shown to downregulate transcriptional activities of NFκB, AP-1 and HIF-1α, with reductions in inflammatory cytokines[58]. Despite these modest anti-inflammatory properties, the statins do not appear to significantly influence either IR or glycemic status. In contrast, high-dose salicylates directly suppress inflammation by targeting NFκB, which improves insulin sensitivity and reduces blood glucose in patients with diabetes[59-61]. The anti-inflammatory properties of TZDs and statins have associated side effects apart from their primary modes of action, thus they may not be safe in the long term. It is necessary to investigate new classes of drugs.

Histone deacetylases (HDACs) are key enzymes that regulate gene expression. Inhibition of histone deacetylase activity has been reported as a new approach to treat diabetes mellitus. Butyrate or trichostatin A, which are histone deacetylase inhibitors, prevented high fat-induced obesity and improved IR in mice[62]. The multiple beneficial effects included: reduced systemic chronic inflammation[63-66], reduced lipid toxicity[67,68], promotion of beta-cell development, proliferation, differentiation and function[69]. Thus HDAC inhibitors may represent a novel drug in the treatment of IR.

CONCLUSION

Hyperglycemia, a commonly exhibited metabolic disorder in critically ill patients, activates the body's inflammatory defense system, causing the cascade release of numerous inflammatory mediators and cytokines, and eventually leads to organ damage. Insulin inhibits hypermetabolism, such as hyperglycemia and lipid degradation, thus could attenuate glucose and FFA-mediated inflammation and improve immunocompetence. More importantly, insulin directly suppresses pro-inflammatory cytokines and induces anti-inflammatory mediators through non-metabolic pathways (Figure 1). Currently, the effects are dependent upon its suppression of innate immune mechanisms and the suppression of transcription factors such as NFκB and Egr-1. With further investigation, the discovery and understanding of the mechanisms underlying the anti-inflammatory effects of insulin opens up the possibility that insulin therapy could be used in multiple clinical practices.

Hyperglycemia, inflammation and IR are inter-related and of reciprocal causation. The relationships between the three entities are far from being elucidated. Hyperglycemia leads to oxidative stress, which further results in inflammation. IR, commonly as a manifestation of hyperglycemia, is pro-inflammatory. Reactive oxygen species is believed to be an important cause of many pathological conditions, including inflammation and IR. It has been established that hyperglycemia is inflammatory whereas insulin is anti-inflammatory. From simple glucose maintenance to the discovery of cardiovascular protection, the knowledge and understanding about insulin is increasing. The pleiotropic effects of insulin including glucose control, and reduction in apoptosis, oxidative/nitrative stress and inflammation, contribute to cardiovascular protection and are beneficial in critical illness. It is not a single effect that mediates the important role of insulin, but it is the whole scenario that promotes its myriad effects. With consistent research, we will gain a better understanding of these working mechanisms, and in doing so, are likely

to find more therapeutic targets and wider applications for this wonder drug.

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P- Reviewers: Collino M, Gómez-Sáez J, Kumar K
S- Editor: Zhai HH **L- Editor**: Cant MR **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

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Role of P2X7 receptors in the development of diabetic retinopathy

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Received: October 22, 2013 Revised: December 4, 2013
Accepted: January 6, 2014
Published online: April 15, 2014

Abstract

The P2X7 receptor is one of the members of the family of purinoceptors which are ligand-gated membrane ion channels activated by extracellular adenosine 5′-triphosphate. A unique feature of the P2X7 receptor is that its activation can result in the formation of large plasma membrane pores that allow not only the flux of ions but also of hydrophilic molecules of up to 900 Da. Recent studies indicate that P2X7-mediated signaling can trigger apoptotic cell death after ischemia and during the course of certain neurodegenerative disorders. Expression of the P2X7 receptor has been demonstrated in most types of cells in the retina. This purinoceptor mediates the contraction of pericytes and regulates the spatial and temporal dynamics of the vasomotor response through cell-to-cell electrotonic transmission within the microvascular networks. Of potential clinical significance, investigators have found that diabetes markedly boosts the vulnerability of retinal microvessels to the lethal effect of P2X7 receptor activation. This purinergic vasotoxicity may result in reduced retinal blood flow and disrupted vascular function in the diabetic retina. With recent reports indicating an association between P2X7 receptor activation and inflammatory cytokine expression in the retina, this receptor may also exacerbate the development of diabetic retinopathy by a mechanism involving inflammation.

Key words: P2X7 receptor; Diabetic retinopathy; Vasotoxicity; Retinal microvessels; Interleukin-1β; Tumor necrosis factor-α

Core tip: This review summarizes the studies regarding the putative role of the P2X7 receptor in triggering purinergic vasotoxicity in the retina and thereby contributing to the progression of diabetic retinopathy.

Original sources: Sugiyama T. Role of P2X7 receptors in the development of diabetic retinopathy. *World J Diabetes* 2014; 5(2): 141-145 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/141.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.141

INTRODUCTION

One of the most important characteristics of diabetic retinopathy (DR) is the death of microvascular pericytes and endothelial cells[1]. The loss of pericytes, contractile cells located on the abluminal wall of capillaries[2], appears to play a critical role in the development of microaneurysms and neovascular tufts[3]. Damage in the endothelial cells can result in a breakdown of the blood–retinal barrier and macular edema[4].

Currently, the mechanisms by which diabetes induces apoptosis in the retinal microvasculature remain uncertain, although oxidative stress, formation of advanced glycation end products, upregulation of protein kinase C, increased polyol pathway flux and focal leukostasis may be taken as important factors[5]. In fact, multiple lethal pathways may be activated during chronic hyperglycemia[6].

Extracellular adenosine 5'-triphosphate (ATP) is an excitatory transmitter both in the peripheral and central nervous systems. P2X receptors are a family of ligand-gated membrane ion channels activated by extracellular ATP. P2X receptors consist of seven isoforms designated

P2X1 to P2X7[7,8]. They are widely distributed in most types of cells in nearly every origin. They are involved in many actions, such as synaptic transmission in the peripheral and central nervous systems, contraction of smooth muscle, platelet aggregation, macrophage activation, cell death and immunomodulation[9,10].

In contrast to other ligand-gated channels in the purinoceptor family, the P2X7 receptor possesses unique features that are likely to be of both physiological and pathophysiological significance. Most importantly, not only does the initial activation of these receptors result in the opening of a non-selective plasma membrane channel, but with sustained activation there is in many types of cells the formation of trans-membrane pores that are permeable to hydrophilic molecules of up to 900 Da[11,12]. Indicative of P2X7 receptors having a role in cell pathology, this receptor has been found to be highly up-regulated in neurons and glial cells located in the ischemic cerebral cortex[13]. P2X7-mediated signaling is also implicated in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and multiple sclerosis[14].

P2X7 RECEPTOR IN THE RETINA

Expression of the P2X7 receptor has been demonstrated in most types of cells in the retina; these include neurons such as the ganglion cells[15,16], as well as glia[17,18] and vascular cells[19]. The P2X7 receptor was found to mediate the contraction of pericytes through an increase in intracellular calcium levels[19]. Interestingly, the spatial and temporal dynamics of this vasomotor response are established by the ability of P2X7 activation to potently inhibit cell-to-cell electrotonic transmission within the retinal microvascular network[19].

In the adult rat retina, immunolabeling for the P2X7 receptor is detected in a number of cells in the inner nuclear layer and ganglion cell layer, suggesting amacrine cells and ganglion cells[15]. This receptor was also found in processes presynaptic to rod bipolar cells, as well as other conventional synapses, suggesting that purines play a role in neurotransmission within the retina and may modulate both photoreceptor and rod bipolar cell responses[20].

In addition to the putative physiological roles of P2X7 receptors, it is reported that stimulation of these receptors can kill retinal ganglion cells *in vitro* and *in vivo* by a mechanism that appears to be dependent on a rise in intracellular Ca^{2+}[21,22]. One of those reports also suggested that the balance between extracellular ATP and its protective metabolite adenosine can influence ganglion cell survival in the living eye[22]. Another study suggested that an early up-regulation of neuronal P2X7 receptors may cause injury of retinal neurons and thereby contribute to the retinal damage[23]. Furthermore, data from our laboratory indicate that the activation of P2X7 receptors is involved in hypoxia-induced death of retinal neurons[24]. Other researchers have indicated mechanical strain triggers ATP release directly from retinal ganglion cells and that this released ATP autostimulates P2X7 receptors. Since extracellular ATP levels in the retina increase with elevated intraocular pressure and stimulation of P2X7 receptors on retinal ganglion cells can be lethal, this autocrine response may exert a deleterious effect on retinal ganglion cells in glaucomatous eyes[25].

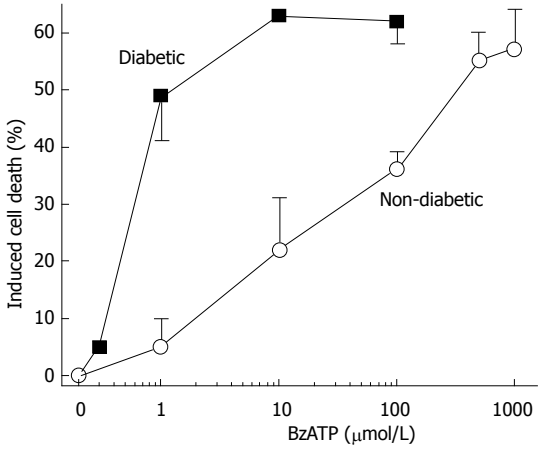

Figure 1 Cell death induced in non-diabetic and diabetic retinal microvessels by the P2X7 agonist, benzoylbenzoyl adenosine triphosphate. From Sugiyama *et al*[30] with permission from Investigative Ophthalmology and Visual Sciences. BzATP: Benzoylbenzoyl adenosine triphosphate.

P2X7 RECEPTOR AND DIABETIC RETINOPATHY

A study showed that human primary fibroblasts in a medium with a high glucose concentration underwent substantial ATP-mediated morphological changes and increased apoptosis. P2X7 was identified as the main purinergic receptor involved in these responses[26]. It has also been reported that fibroblasts from type 2 diabetes patients are characterized by a hyperactive purinergic loop based either on a higher level of ATP release or on increased P2X7 reactivity[27]. Another study revealed that changes in Müller cell membrane conductance in proliferative diabetic retinopathy (PDR), *i.e.*, the down-regulation of active Kir channels and the membrane depolarization, likely disturb voltage-dependent Müller cell functions, such as regulation of local ion concentrations and uptake of neurotransmitters[28]. The enhanced entry of calcium ions from the extracellular space and the subsequent stimulation of calcium-activated potassium channels may trigger Müller cell proliferation in PDR. Others reported that prolonged stimulation of the P2X7 receptor elicited permeabilization exclusively in microglial cells but not in neurons of the inner retina[29].

Our experiments, using pericyte-containing retinal microvessels, have shown a diabetes-induced increase in the vulnerability of retinal microvessels to the lethal effect of P2X7 receptor activation[30]. In other words, the agonist concentration needed to open large membrane pores and trigger apoptosis decreased markedly soon after the onset of streptozotocin-induced hyperglycemia in rats (Figure 1). It was also found that extracellular nicotinamide adenosine dinucleotide (NAD^+) caused cell death in the retinal microvasculature by a mechanism involving the

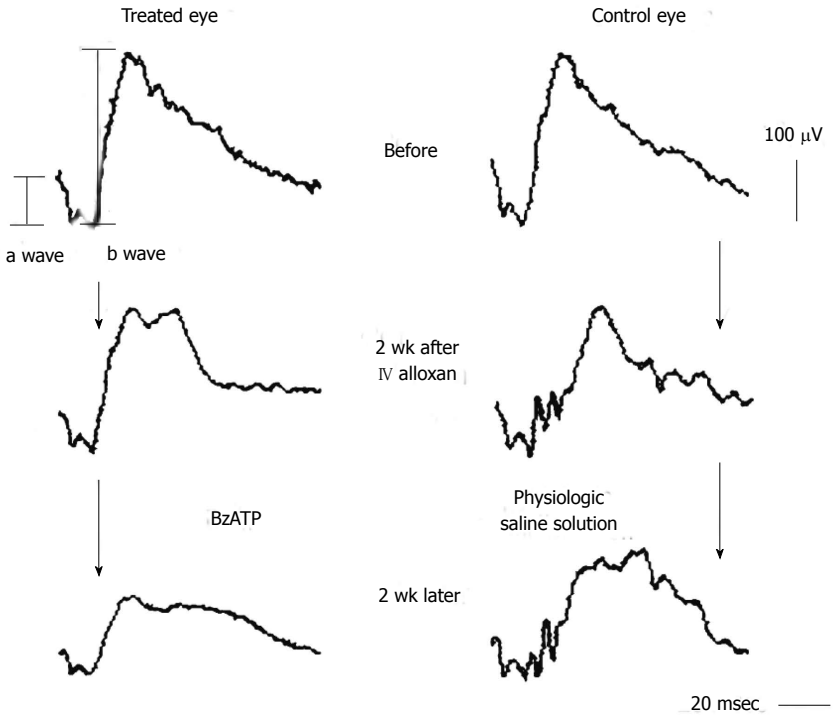

Figure 2 Typical changes of electroretinography after intravitreal injection (Ⅳ) of benzoylbenzoyl adenosine triphosphate (50 nmol) or physiological saline solution in an alloxan-induced diabetic rabbit. The amplitudes of a and b waves and oscillatory potentials were reduced in the BzATP-treated eye. From Sugiyama *et al*[32] with permission from Archives of Ophthalmology. BzATP: Benzoylbenzoyl adenosine triphosphate.

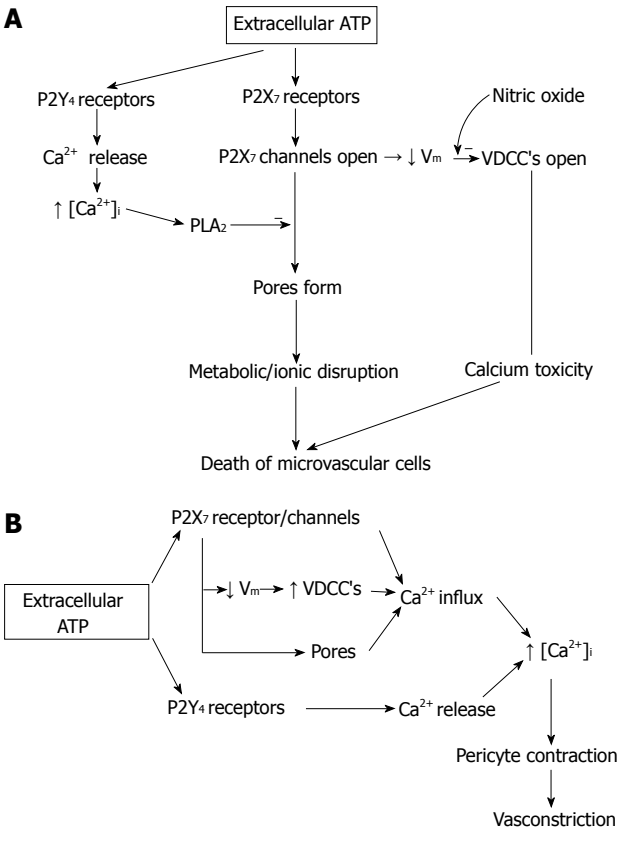

Figure 3 Models of the physiological and pathobiological effects of adenosine 5'-triphosphate in the retinal microvasculature. A: Putative mechanisms regulating purinergic vasotoxicity; B: Putative mechanisms by which extracellular adenosine 5'-triphosphate (ATP) causes pericyte Ca^{2+} levels to rise and thereby the contraction of these mural cells and the constriction of adjacent lumens. From Sugiyama *et al*[33].

activation of the P2X7 purinoceptor and the formation of transmembrane pores. Soon after the onset of diabetes, the sensitivity of retinal microvessels to the vasotoxic effect of extracellular NAD^+ increased by approximately 100-fold[31]. In our *in vivo* study using the laser speckle circulation analyzer and electroretinography, soon after the onset of alloxan-induced diabetes, retinal blood velocity and function become more vulnerable to reduction initiated through the P2X7 receptor (Figure 2)[32]. Additional investigations indicate that, under physiological conditions, the formation of P2X7 pores is tightly regulated *via* a nitric oxide- and P2Y4-dependent pathway that limits the rise in pericyte calcium during the activation of these purinoceptors[33]. However, if this regulatory mechanism becomes dysfunctional, as appears to occur in the diabetic retina (Figure 3)[33], then purinergic vasotoxicity may contribute to the microvascular cell death that is a hallmark of DR.

Of additional interest, recent studies of DR in experimental models suggest the P2X7 receptors may have a role in mediating cytokine-induced vascular inflammatory reactions that can degrade the integrity of the blood-retinal barrier and thereby contribute to retinal vascular occlusion and ischemia[34]. More specifically, there are a number of reports linking P2X7 receptor activation in the retina with the expression of inflammatory cytokines[35]. For example, P2X7 agonists enhance the release of interleukin (IL)-1β and tumor necrosis factor (TNF)-α from hypoxia-activated retinal microglia[17]. In addition, our recent data suggest that the up-regulation of TNF-α, IL-1β and IL-6 may be involved in the retinal ganglion cell death that can occur with P2X7 receptors activated after an elevation in the intraocular pressure[36]. Although it is clear that more investigation is needed, these new findings further suggest that this purinoceptor may have a role in the progression of DR.

In conclusion, a variety of recent experimental stud-

ies are providing evidence that the P2X7 purinoceptor is a potential therapeutic target of a pharmacological strategy designed to diminish or prevent cell death in the diabetic retina.

ACKNOWLEDGMENTS

I would like to express my appreciation to Professor Donald G. Puro for leading me to this research topic during my stay in 2001 as a research fellow in his laboratory at the University of Michigan as well as for kindly editing this manuscript, and also to Professor Tsunehiko Ikeda for giving me the opportunity to study abroad and then to continue investigating this topic in Department of Ophthalmology at Osaka Medical College.

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P- Reviewers: Gurunathan S, Koleva-Georgieva DN, Le YZ
S- Editor: Ma YJ **L- Editor**: Roemmele A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diabetes and cancer: Associations, mechanisms, and implications for medical practice

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Supported by Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents; Program for Zhejiang Leading Team of Science and Technology Innovation; Fundamental Research Funds for the Central Universities
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Received: November 28, 2013 Revised: February 7, 2014
Accepted: April 17, 2014
Published online: June 15, 2014

Abstract

Both diabetes mellitus and cancer are prevalent diseases worldwide. It is evident that there is a substantial increase in cancer incidence in diabetic patients. Epidemiologic studies have indicated that diabetic patients are at significantly higher risk of common cancers including pancreatic, liver, breast, colorectal, urinary tract, gastric and female reproductive cancers. Mortality due to cancer is moderately increased among patients with diabetes compared with those without. There is increasing evidence that some cancers are associated with diabetes, but the underlying mechanisms of this potential association have not been fully elucidated. Insulin is a potent growth factor that promotes cell proliferation and carcinogenesis directly and/or through insulin-like growth factor 1 (IGF-1). Hyperinsulinemia leads to an increase in the bioactivity of IGF-1 by inhibiting IGF binding protein-1. Hyperglycemia serves as a subordinate plausible explanation of carcinogenesis. High glucose may exert direct and indirect effects upon cancer cells to promote proliferation. Also chronic inflammation is considered as a hallmark of carcinogenesis. The multiple drugs involved in the treatment of diabetes seem to modify the risk of cancer. Screening to detect cancer at an early stage and appropriate treatment of diabetic patients with cancer are important to improve their prognosis. This paper summarizes the associations between diabetes and common cancers, interprets possible mechanisms involved, and addresses implications for medical practice.

Key words: Diabetes mellitus; Cancer; Association; Mechanism; Medical practice

Core tip: The diabetes-cancer link is summarized and discussed in detail and it may potentially be attributed to hormonal disorders, chronic inflammation and metabolic alterations. Besides, implications for medical practice are also addressed.

Original sources: Xu CX, Zhu HH, Zhu YM. Diabetes and cancer: Associations, mechanisms, and implications for medical practice. *World J Diabetes* 2014; 5(3): 372-380 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/372.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.372

INTRODUCTION

The prevalence of diabetes mellitus (DM) is increasing worldwide. According to the estimates by the International Diabetes Federation, the global prevalence of type

2 diabetes mellitus (T2DM) is 8.3%. The prevalence of T2DM varies by country and area. The highest rate is 10.5% in North America, 8.7% in South-East Asia, 6.7% in Europe and 4.3% in Africa. It is predicted that 552 million people worldwide will develop diabetes by 2030[1].

DM and cancer are frequently diagnosed in the same individual[2]. DM is reported to be associated with an increased risk of different types of cancer, including pancreatic, liver, breast, colorectal, urinary tract, gastric, and female reproductive cancers. The relative risk ranges from 2.0 to 2.5 for liver, pancreatic and endometrial cancers, and 1.2 to 1.5 for breast, colon and bladder cancers associated with DM[3]. It is worth noting that DM is a growing health problem worldwide. Even if the increased risk in cancer incidence and mortality due to DM is small, the consequence would be significant at the population level[4].

The mechanism of DM associated with cancer remains uncovered and needs to be examined in further studies. The mechanism for the diabetes-cancer link has been hypothesized to be mainly related to hormonal [insulin and insulin-like growth factor (IGF)-1], inflammatory or metabolic (hyperglycemia) characteristics of the DM and even to certain treatments[5]. Anti-diabetic medications may have effects on the risk for cancer. Increasing evidence shows that insulin sensitizers such as metformin and thiazolidinediones (TZDs) are associated with prostate cancer[6] and HER2-positive breast cancer[7] among diabetic patients. The diabetic patients who are treated with insulin or insulin secretagogues are more likely to develop cancer than those with metformin[8-11].

In this paper, we summarize the associations between diabetes and cancer in epidemiologic studies, possible mechanisms and implications for medical practice.

POSSIBLE BIOLOGIC LINKS BETWEEN DIABETES AND CANCER RISK

Insulin resistance

Insulin resistance is very common in T2DM, in which circulating insulin level is frequently increased. The insulin/IGF axis plays an important role in diabetes-associated increased risk and progression of cancer. The cancer cells overexpress insulin and IGF-1 receptors[2].

Hyperinsulinemia is a hallmark of insulin resistance. The mechanisms whereby hyperinsulinemia could link diabetes and cancer have been extensively investigated and discussed. Hyperinsulinemia may influence cancer development through ligand by binding with the insulin receptor (IR) and/or indirectly through increasing circulating IGF-1 levels[12]. Insulin signal transduction is mediated through two IR isoforms: IR-A and IR-B[13]. IR-A recognizes insulin and IGFs, with a higher affinity for IGF2 than IGF1, and IR-B is insulin specific and is mainly involved in glucose homeostasis. Insulin binds with IR-A and exerts a direct pro-growth mitogenic effect. When elevated, insulin can increase the hepatic expression of IGF-1 and then activate the IGF-1 receptor, further stimulating cell growth through this mechanism[14,15]. IR-A and IGF-1 receptor are expressed primarily in fetal tissues and cancer cells[16].

The independent role of the IR is confirmed by the observation that down-regulation of IRs in LCC6 cells reduces xenograft tumor growth in athymic mice and inhibits lung metastasis[17]. Besides, blockade of the IGF-1 receptor has been associated with decreased growth of breast cancer cells[18,19]. Hyperinsulinemia also results in decreased levels of IGF binding protein-1 and thus increased levels of bioactive IGF-1[20,21].

Multiple downstream signaling pathways are activated after IRs or IGF-1 receptors interact with their ligands. By phosphorylation of adaptor proteins, two major pathways are involved: (1) the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), PI3K/Akt/forkhead box O, and Ras/MAPK/extracellular signal-related kinase 1/2 pathway which plays important roles in cancer cell growth and carcinogenesis[22,23] is activated; and (2) the inhibitor of the oncogenic β-catenin signaling (glycogen synthase kinase 3β) is inactivated, through the PI3K/Akt signaling pathway, resulting in β-catenin signaling activation that has been related to cancer stem cells and chemoresistance[24].

Hyperglycemia

Hyperglycemia has been classically considered as a subordinate whereas hyperinsulinemia as a primary causal factor for cancer[25].

Several large cohort and case-control studies have found a positive relationship between hyperglycemia and the risk of cancer[26-29]. In a tumor-prone animal model, it was found that the number and size of liver tumors increased and apoptosis was reduced in insulin-deficient hyperglycemic mice compared with insulin-sufficient mice. This phenomenon was reversed by insulin therapy[30]. However, *in vivo* studies showed that T1DM, which is characterized by hyperglycemia, reduces the tumor growth. This finding does not support that hyperglycemia increases tumor growth, at least in the setting of insulin deficiency[31]. A recent research found that tumors continue to consume high amounts of glucose, regardless of plasma glucose levels[32]. A recent meta-analysis confirmed this finding that improved glycemic control does not reduce cancer risk in diabetic patients[33]. Hyperglycemia may be an independent risk factor for cancer. Further studies are needed to evaluate the relative roles of insulin and glucose.

The possible mechanisms of hyperglycemia increasing cancer risk include "indirect effect" and "direct effect"[34]. The "indirect effect" is the action that takes place at other organs and will later on influence tumor cells by inducing production of circulating growth factors (insulin/IGF-1) and inflammatory cytokines. The "direct effect" is the effect that is exerted directly upon tumor cells by increasing proliferation, inducing mutations, augmenting invasion and migration and rewiring cancer-related signaling pathways. Recently, Wnt/β-catenin signaling has been suggested as a key cancer-associated pathway and

Table 1 Combined relative risk and 95%CI in meta-analyses of cohort studies of cancer risk in different organs of diabetic patients

Cancer	Ref.	No. of cohort studies	RR (95%CI)	RR (95%CI) male	RR (95%CI) female
Pancreas	Ben *et al*[76], 2011	35	1.94 (1.66-2.27)	1.70 (1.55-1.87)[1]	1.60 (1.43-1.77)[1]
Liver	Wang *et al*[56], 2012	18	2.01 (1.61-2.51)	1.96 (1.71-2.24)[1]	1.66 (1.14-2.41)[1]
Breast	De Bruijn *et al*[66], 2013	20	1.23 (1.12-1.34)	NA	1.23 (1.12-1.34)
Endometrium	Zhang *et al*[67], 2013	15	1.81 (1.38-2.37)	NA	1.81 (1.38-2.37)
Colon-rectum	Jiang *et al*[62], 2011	30	1.27 (1.21-1.34)	1.25 (1.17-1.33)[1]	1.23 (1.13-1.33)[1]
Kidney	Bao *et al*[70], 2013	11	1.39 (1.09-1.78)	1.28 (1.10-1.48)	1.47 (1.18-1.73)
Bladder	Zhu *et al*[73], 2013	29	1.29 (1.08-1.54)	1.36 (1.05-1.77)	1.28 (0.75-2.19)
Prostate	Zhang *et al*[78], 2012	25	0.92 (0.81-1.05)	0.92 (0.81-1.05)	NA
Gastric	Yoon *et al*[81], 2013	11	1.20 (1.08-1.34)	1.10 (0.97-1.24)	1.24 (1.01-1.52)
Non-Hodgkin's lymphoma	Castillo *et al*[85], 2012	11	1.21 (1.02-1.45)	1.13 (0.96-1.34)	1.24 (0.97-1.58)

[1]Based on the studies reported by gender. NA: Unavailable.

high glucose enhances this signaling pathway by allowing nuclear retention and accumulation of transcriptionally active β-catenin independently of hyperinsulinemia, adipokines or inflammation[35,36].

Chronic inflammation

The deregulated metabolism in poorly controlled diabetes causes a long-term pro-inflammatory condition characterized by increased levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), C-reactive protein, and other markers of chronic inflammation. Emerging evidence suggests that persistent inflammation can promote genetic instability and chronic inflammation is associated with increased cancer risk[37-40]. This finding is also supported by the classical evidence that non-steroidal anti-inflammatory drugs can reduce the risk of certain cancers[41-44].

Tumor-promoting mechanism of inflammation in diabetic patients is not much clear. Chronic inflammation and chronic oxidative stress go hand-in-hand. Oxidants affect almost all stages of the inflammatory response process, including the release of inflammatory cytokines, the sensing by innate immune receptors from the families of Toll-like receptors and the nucleotide-binding oligomerization domain-like receptors, and the activation of signaling initiating the adaptive cellular response to such signals[40]. Reactive oxygen species can cause damage to lipids, protein and DNA, and then initiate carcinogenesis[45-47]. Meanwhile, chronic inflammation is associated with high levels of TNF-α, which would strongly activate nuclear factor-kappa B (NF-κB) and further induce downstream signaling transduction to promote the development and progression of many tumors. NF-κB is involved in the proliferation and survival of malignant cells, promotes angiogenesis and metastasis, subverts adaptive immunity, and mediates responses to hormones and/or chemotherapeutic agents[48-50]. Therefore, continued exposure to chronic inflammation and oxidative stress puts susceptible cells at risk of progression toward malignant transformation[31].

IMPACT OF DIABETES ON CANCER

Evidence from animal studies

DM is mainly characterized by insulin resistance, hyperinsulinemia, hyperglycemia, and dyslipidemia. The independent role of diabetes and obesity in caner development has been difficult to distinguish since obesity is also related to inflammation and hyperinsulinemia. Studies in transgenic diabetic mice might shed light on the relative contributions of these factors. In a transgenic model of skin and mammary carcinogenesis, non-obese diabetic mice (A-ZIP/F-1) developed more tumors than wild-type controls[51]. In MKR mouse models of mammary carcinogenesis, female mice with T2DM showed accelerated mammary gland development and breast cancer progression independent of obesity and inflammation[52]. Hyperinsulinemia promoted the growth of primary mammary tumor and subsequent metastasis to the lung[53]. Tumor progression was abrogated with the decreased level of serum insulin after treatment with anti-insulin drugs[54]. Taken together, findings from animal studies support that diabetes plays interconnected roles in the development of cancer.

Epidemiologic findings

The findings from a meta-analysis of 12 cohort studies showed that diabetes increased the risk of all-cancer incidence for overall subjects, with a pooled adjusted RR of 1.14 (1.06-1.23) for men, and 1.18 (1.08-1.28) for women[55]. Diabetes is reported to be associated with several types of cancer, including pancreas, liver, breast, colorectal, urinary tract, gastric, and female reproductive cancers. Meta-analyses on the associations between diabetes and site specific cancer are summarized in Table 1.

Liver cancer: In various studies examining the link between DM and cancer, the highest risk has been seen for liver cancer. A meta-analysis demonstrated that individuals with diabetes had a 2.0-fold increased risk of developing hepatocellular carcinomas (HCC), compared with non-diabetics. And this link was observed in both men and women[56]. The liver is exposed to high concentrations of endogenously produced insulin transported *via* the portal vein. Hyperinsulinemia stimulates the production of IGF-1, which further promotes cellular proliferation and then inhibits apoptosis in the liver. The important role of hyperinsulinemia and IGF-1 in hepatic carcinogenesis has been demonstrated by *in vitro*, *in vivo*, and epidemiologic studies[57,58]. Liver steatosis, hepatitis,

and cirrhosis are more frequent among diabetic patients and are well known risk factors for HCC. Insulin resistance stimulates the release of multiple pro-inflammatory cytokines and consequently promotes the development of hepatic steatosis and inflammation and subsequent cancer in the liver[59]. A causal relationship was also reported by Jee *et al*[60], who found that fasting glucose and liver cancer risk had a dose-responsive relationship. Besides, T2DM-induced hyperglycemia induces the release of TNF-α and IL-6 in patients with hepatic steatosis and enhances the pathogenesis of cancer[61].

Colorectal cancer: A meta-analysis comprising 30 cohort studies showed that diabetes was associated with an increase in the risk of colorectal cancer, with a combined RR of 1.27 (1.21-1.34). This association was consistent for both men and women[62]. Our previous retrospective cohort study showed that a significant association of diabetes was found with colon cancer and not with rectal cancer[63]. This finding indicated that there was a subsite specific association of T2DM with colorectal cancer. General factors like hyperinsulinemia and IGF-1 have contributed to intramucosal adenocarcinomas. Diabetic patients have slower bowel peristalsis and more common constipation and thus increased exposure to bowel toxins (*i.e.*, elevated concentrations of fecal bile acids) and potential carcinogens[64]. Animal models have demonstrated that increased concentrations of fecal bile acids could induce colorectal carcinogenesis[64,65].

Breast and other female cancers: A meta-analysis including 20 cohort studies found an association between diabetes and breast cancer with a summary RR of 1.23 (1.12-1.34)[66]. A meta-analysis including 15 cohort studies reported an increased risk [RR = 1.81 (1.38-2.37)] of endometrial cancer in diabetic women[67]. Hyperinsulinemia could increase the levels of bioactive estrogens by reducing the concentration of circulating sex hormone binding protein in diabetic women. It is well known that bioactive estrogens are the risk factors for malignancies of female reproductive organs[68,69]. Increased bioactive estrogen will stimulate the proliferation of breast and endometrial cells and the inhibition of apoptosis to increase cancer risk.

Kidney and bladder cancers: A meta-analysis including eleven cohort studies showed that diabetes was significantly associated with an increased risk of kidney cancer [RR = 1.39 (1.09-1.78)]. The association was slightly stronger in women [RR = 1.47 (1.18-1.83)] than in men [RR = 1.28 (1.10-1.48)][70]. Hypertension and late stage renal disease, two common comorbidities of DM, contribute to the increased incidence of kidney cancer[71,72]. Impaired renal function results in higher circulating levels of carcinogens and toxins and immune inhibition and thereby renders the kidney susceptible to carcinogens and tumor growth. Findings from a meta-analysis of 29 cohort studies suggest that individuals with DM display an increase in the risk of bladder cancer [RR = 1.29 (1.08-1.54)]. The positive association is only observed in men [RR = 1.36 (1.05-1.77)][73]. In addition to general factors, the frequent infections of the urinary tract in diabetic patients might also be involved[74].

Pancreatic cancer: In a 3-year follow-up study[75], subjects with new-onset DM had a higher risk of pancreatic cancer with a RR of 7.94 than the subjects without DM. A meta-analysis of 35 cohort studies showed that DM was associated with an increased risk of pancreatic cancer in both men and women[76]. However, the question arises about whether diabetes is a risk factor or the consequence of the pancreatic cancer (so-called "reverse causality"). Pancreatic cancer might induce a diabetic status because of impaired pancreatic beta cells. *In vitro* studies show that blockage of insulin receptors and impaired insulin action and glucose transport in a model of pancreatic cancer led to insulin resistance[77]. However, the new onset of pancreatic cancer induced DM depends on the peripheral insulin resistance rather than on the impaired pancreatic beta cells. On the other hand, in patients with T2DM exocrine pancreatic cells are exposed to very high insulin levels because of their proximity to insulin secreting islets. Insulin stimulates the growth of cancer cells. Thus, hyperinsulinemia might account for the risk of developing pancreatic cancer in T2DM.

Prostate cancer: Prostate cancer risk appears to decrease in patients with diabetes. An inverse association was observed between diabetes and risk of prostate cancer in the studies from the United States but not in the studies from other countries, as shown by an updated meta-analysis[78]. The protective effect of DM was also observed in different grades or stages of prostate cancer in another meta-analysis[79]. One possible explanation is that low testosterone levels have been shown in diabetic men. The conversion of testosterone to dihydrotestosterone promotes prostate cell growth[80].

Other cancers in diabetes: A 20% increased gastric cancer risk in diabetic patients was found in a meta-analysis. A positive association was observed in female diabetic patients, whereas it was not the case in diabetic men[81]. The IGF/IGF-IR axis interacts with the vascular endothelial growth factor/vascular endothelial growth factor receptor system in gastrointestinal malignancies[82,83]. It is also possible that reactive oxygen-dependent DNA damage further enhances the effect of *Helicobacter pylori* on epithelial cell proliferation[84]. A meta-analysis of large prospective cohort studies has shown a moderate increase of non-Hodgkin's lymphoma in diabetic patients, whereas stratified analysis by gender shows no significance based on the studies with reported cancer incidence by gender[85]. The immune dysfunction related to impaired neutrophil activity and abnormalities in cellular and humoral immunity in diabetes may contribute to cancer development[86].

MORTALITY

A meta-analysis suggests that preexisting diabetes is as-

Table 2 Pooled HRs and 95%CI of all-cause mortality in cancer patients with and without preexisting diabetes mellitus

Cancer	Ref.	No. of cohort studies	HR (95%CI)	HR (95%CI) male	HR (95%CI) female
Pancreas	Barone *et al*[87], 2008	4	1.09 (0.70-1.69)	NA	NA
Liver	Wang *et al*[56], 2012	3	1.56 (1.30-1.87)	1.84 (1.34-2.51)	1.31 (1.06-1.61)
Breast	De Bruijn *et al*[66], 2013	20	1.38 (1.20-1.58)	NA	1.38 (1.20-1.58)
Endometrium	Zhang *et al*[67], 2013	6	1.23 (0.80-1.90)	NA	1.23 (0.80-1.90)
Colon-rectum	Jiang *et al*[62], 2011	11	1.20 (1.03-1.40)	1.26 (1.04-1.52)	1.18 (0.98-1.41)
Kidney	Bao *et al*[70], 2013	8	1.12 (0.99-1.20)	NA	NA
Bladder	Zhu *et al*[73], 2013	11	1.33 (1.14-1.55)	1.54 (1.30-1.82)[1]	1.50 (1.05-2.14)[1]
Prostate	Barone *et al*[87], 2008	3	1.51 (0.94-2.43)	1.51 (0.94-2.43)	NA
Gastric	Tian *et al*[88], 2012	NA	1.29 (1.04-1.59)	NA	NA
Non-Hodgkin's lymphoma	Lin *et al*[89], 2007	1	1.33 (0.61-2.90)	NA	NA

[1]Based on the studies reported by gender. NA: Unavailable.

sociated with a higher risk of all-cause long term cancer mortality compared with non-diabetic individuals HR = 1.41 (1.28-1.55)[87]. Mortality among diabetes was significantly increased for liver, breast, and bladder cancers, with pooled RRs of 1.56 (1.30-1.87)[56], 1.38 (1.20-1.58)[66], and 1.33 (1.14-1.55)[73], respectively. Similar but mild results are also seen in gastric cancer[88] and colorectal cancer[62]; with 29% and 20% increased all-cause mortalities, respectively (Table 2). Non-significance is found for the cancers of the pancreas[87], prostate[87], kidney[70], endometrium[67], and non-Hodgkin's lymphoma[89] (Table 2).

Several possible explanations might elucidate the increased risk of cancer death in DM. Impaired immune function and pro-inflammatory condition in diabetes may make the cancer more aggressive, favor cancer growth by making host organism less resistant to cancer progression, and strengthen the metastatic potential of cancer. Hyperglycemia may be an important risk factor. There is evidence that poor glycemic controls can lead to poorer outcomes. Survival rates in cancer are decreasing linearly with declining glycemic controls[90]. Diabetic patients may have a worse response to chemotherapy with a higher occurrence of adverse effects compared with non-diabetic individuals.

Diabetes patients are more often poor candidates for surgery. Preexisting diabetes was associated with increased odds of postoperative mortality across all cancer types [OR = 1.51 (1.13-2.02)][91].

IMPLICATIONS FOR MEDICAL PRACTICE

Cancer screening is required for patients with preexisting diabetes

As shown by the above studies, patients with DM have a higher risk of developing certain types of cancer. A healthy diet, physical activity, and weight management could decrease the risk and improve outcomes of DM and some types of cancer. This was supported by a consensus report of the American Diabetes Association and the American Cancer Society[2]. In order to improve the prognosis, early screening of DM-related cancers is important for T2DM patients. Cancer screening tests of proven benefit for malignancies (breast, colon, endometrial cancer, *etc.*) in at-risk individuals/populations should begin relatively earlier than the general population. Future cancer screenings should be based on current existing recommendations. However, specific DM-related cancer screening recommendations remain to be made.

The impact of anti-diabetic treatments on cancer risk

The major classes of DM drugs function to replace circulating insulin and reduce hyperglycemia by different mechanisms or to reduce the associated obesity[92]. Insulin sensitizers, including metformin and TZDs, are oral antidiabetic drugs that decrease insulin resistance by altering signaling through the AKT/mTOR pathway[93,94].

Metformin has been used with confidence in the treatment of T2DM[95]. Emerging evidence from research on humans and from the preclinical setting suggests that metformin has an anti-cancer effect. A meta-analysis of 17 randomized controlled trials showed a clinically significant 39% decreased risk of cancer with metformin use in patients with or at risk for diabetes, compared to no use of metformin[96]. Metformin can decrease cell proliferation and induce apoptosis in certain cancer cell lines[97,98]. In a recent retrospective cohort study, metformin use is not associated with improved survival in subjects with advanced pancreatic cancer[99]. Whereas metformin use was also reported to be associated with a lower risk of colon, liver, pancreas, or breast cancers, it was not associated with the risk of prostate cancer[100,101]. In a meta-analysis by Colmers *et al*[102], TZD-based therapy has been associated with a potential cancer risk, primarily pioglitazone with bladder cancer, as well as a protective role in breast, lung, and colorectal cancers. In combination, the majority of studies showed that metformin therapy decreases and insulin and insulin secretagogues slightly increase the risk of certain cancers in T2DM. Nonetheless, it is premature to prescribe metformin and TZDs solely for those as yet unproven indications for cancers.

Managing diabetic patients with cancer

Managing diabetes can be a daunting task for patients with cancer. Diabetes may negatively impact both cancer risk and outcomes of cancer treatment. It is clear that comorbidities may play a role in clinical outcomes in patients with cancer. Clinicians who treat cancer patients with T2DM should pay more attention to comorbidi-

ties. Thus, rigorous and multifactorial approaches should be adopted to control diabetes for patients undergoing treatment for malignancies. Poor glycemic control increases morbidity and mortality in patients with cancer. Therefore, hyperglycemia management in patients with cancer is important. Monitoring symptoms of both hyperglycemia and hypoglycemia is necessary. DM patients with cancer and their family members should monitor these symptoms and render suitable medical treatment once these symptoms occur. For hospitalized patients with acute concurrent complications, aggressive glycemic management should be taken to improve the prognosis.

CONCLUSION

Previous evidence provides strong support for an increase of both cancer risk and mortality in diabetic patients and more evidence for certain site-specific cancers. The molecular mechanisms for the association between diabetes and cancer development are still uncovered. As underlined in this review, mechanisms on hormonal (insulin and IGF-1), inflammatory and metabolic (hyperglycemia) characteristics have been proposed to elucidate this association. Guidelines specific for diabetic patients should include both treatment in medical practices and mass screening for specific cancers according to the risk factor profile of each patient.

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P- Reviewers: Pirola L, Xu H **S- Editor**: Wen LL
L- Editor: Wang TQ **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Domino effect of hypomagnesemia on the innate immunity of Crohn's disease patients

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Author contributions: All authors contributed equally to the preparation and revision of the manuscript.

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Received: May 15, 2014 Revised: May 28, 2014
Accepted: May 29, 2014
Published online: August 15, 2014

Abstract

Digestive diseases play major role in development and complications of other disorders including diabetes. For example, Crohn's disease (CD) is an inflammatory bowel disease associated with Mycobacterium avium subspecies paratuberculosis. The inflammation is a complex process that involves the activity of both innate and adaptive immune responses. CD lesions are primarily due to T cell response, however; innate immune response has a significant role in initiating its pathogenesis. Toll-like receptors and NOD-like receptors promote the activity of nuclear factor (NF)-κB pathway for cytokines production. This results in the production of high levels of tumor necrosis factor-α, interleukin (IL)-1β and IL-6. Moreover, intestinal inflammation of CD is related to increased activity of NMDA receptors and the release of substance P. Imbalanced magnesium homeostasis in CD is a frequent finding in CD, Diabetes and others. The loss of such a major mineral affects many physiological processes in the body including its role as an immunomodulator. This review aims to (1) describe the significance of hypomagnesemia in the release of pro-inflammatory mediators in CD; (2) demonstrate effects of magnesium on pathways like NF-κB; (3) address the role of hypomagnesemia in the activity of CD; and (4) examine possible future research to establish a standard magnesium supplementation strategy; helping patients with CD or other disorders to maintain a sustained remission.

Key words: Diabetes; Crohn's disease; Hypomagnesemia; Inflammatory bowel disease; Mycobacterium paratuberculosis

Core tip: Magnesium is an essential trace mineral, which plays key role as an immunomodulator in many pathways leading to homeostasis. Hypomagnesemia is common in patients with Crohn's disease (CD) and may be the cause of upregulation of pro-inflammatory factors leading to aggravating symptoms. Therefore, understanding the role of magnesium in maintaining a healthy immune response is important for effective treatment of patients with CD.

Original sources: Naser SA, Abdelsalam A, Thanigachalam S, Naser AS, Alcedo K. Domino effect of hypomagnesemia on the innate immunity of Crohn's disease patients. *World J Diabetes* 2014; 5(4): 527-535 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/527.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.527

INTRODUCTION

Inflammatory bowel disease (IBD) generally describes a group of conditions sharing the characteristic of chronic inflammation of the gastrointestinal tract. The two most common conditions in this category are ulcerative colitis (UC) and Crohn's disease (CD)[1]. In both conditions, the immune system is mistaken food particles and normal flora for foreign materials[2,3]. This will induce an im-

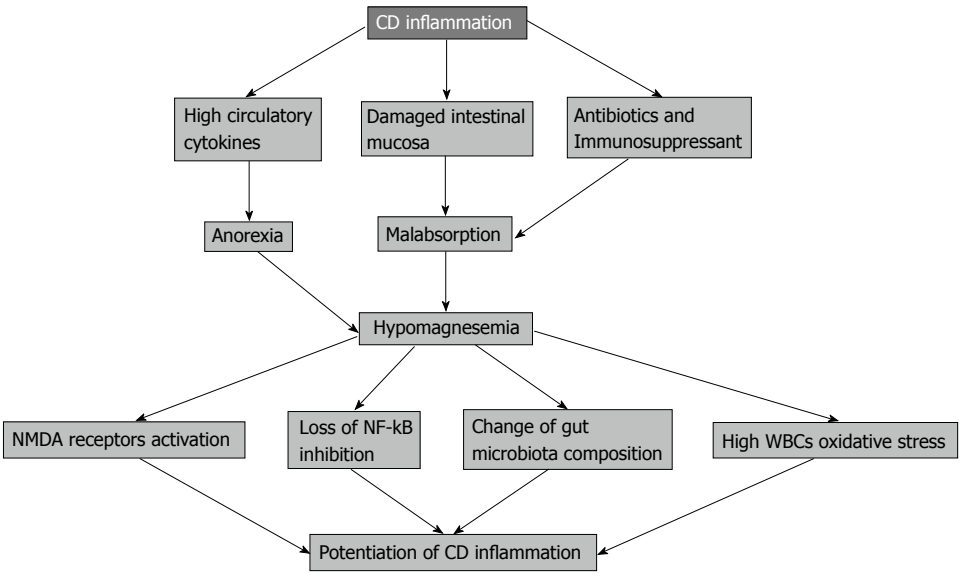

Figure 1 Role of hypomagnesaemia in Crohn's disease complications.

mune response attracting the leukocytes to infiltrate the intestine. The result is destruction of intestinal mucosal cells leading to a state of chronic inflammation. Its distribution and involvement varies between UC and CD. UC is usually confined to the colon while CD can affect any site throughout the gastrointestinal tract from mouth to anus[4]. As both conditions progress, continuation of lesions in the colon becomes a characteristic for UC[5], whereas skipping some locations in the gastrointestinal tract or regional enteritis becomes a characteristic for CD[6]. Moreover, small bowels and the beginning of the large bowels are commonly affected in CD[1]. This difference in lesion locations contributes to the variations in the clinical presentation of UC and CD, as well as the severity of complications.

According to the Center for Disease Control and Prevention, both sexes are equally susceptible to IBD[7], with a majority of the affected population in between their 10 to 30 years of age[8]. Among the most susceptible are Caucasian and Ashkenazi Jewish origins[9]. As gold standard diagnosing criteria for IBD are lacking and the condition gets frequently misclassified, precise incidence and prevalence rates are limited. However, both conditions are noted to be at its highest rate of new diagnoses in industrialized North America and Europe for CD and UC, respectively[8]. In the United States, an estimated 1.4 million individuals suffer from IBD[3,7], of which 20.2 per 100000, per person years suffer from CD[8]. Although the etiology for IBD has not been well established, genetic components[4,8], diet, and environmental factors such as smoking[3] are associated with an increased risk of pathogenesis. Nevertheless, the impact of IBD in the United States creates a huge burden in the health care system, especially for CD with an estimated cost of $2.29 billion annually[10].

In particular, the high cost in CD can be attributed to therapeutic management, physician visits, and hospital stays because of the chronic nature and recurrence of the disease[10]. A detailed review of factors that can influence the persistence of CD might lead to establishing therapeutic strategies that can maintain remission for relatively longer periods. One of these factors includes nutritional deficiency due to malabsorption of vitamins and minerals such as magnesium, a frequent finding in CD patients particularly during high activity of the disease[11-15]. Magnesium deficiency or hypomagnesemia is very understudied and underestimated especially when it comes to its relation to CD (Figure 1). This finding encourages further research about the role of magnesium in inflammation and the possibility of linking this to the breakouts of the CD.

This review aims to (1) describe the significance of low magnesium levels in the release of pro-inflammatory mediators like interleukin 1 (IL-1), IL-6, tumor necrosis factor (TNF)-α as CRP levels; (2) demonstrate effects of magnesium on inflammatory response pathways like nuclear factor (NF)-κB; (3) address the role of hypomagnesemia in the activity of CD as one of the associated nutritional deficiencies; and (4) examine possible future research to establish a standard magnesium supplementation strategy to CD patients maintaining remission for relatively longer periods.

INFLAMMATION IN CD: INNATE *VS* ADAPTIVE

CD is primarily a T cell autoimmune disorder[16,17], however; innate immune response has a significant role in its pathogenesis, as we will demonstrate in this paper (Table 1). Traditionally, T helper 1 (Th1) cells were believed to be the main immune cells responsible for most of the intestinal tissue damage in CD[17,18]. Th1-related cytokines like interferon (IFN)γ, which acts as the major inflammatory mediator in CD[19], are released as a response to Th1 stimulation by IL-12 from naïve T cells[19,20]. Therefore, it was plausible to think that it is possible to control CD

Table 1 Key immune effectors for Crohn's disease inflammatory processes

	Role	Ref.
Toll-like receptors	Their overexpression promotes NF-κB pathway leading to immune intolerance to gut normal flora	[17,24,25,28]
IL-1 TNF-α	Stimulation of intestinal stromal cells to release matrix metalloproteinases leading to mucosal damage	[17,20]
Th1 Th17 cells	Secretion of IFNγ, IL-17 and IL-21 activating macrophages (MØ) and stromal cells to release MMPs	[4,17]
NMDA receptors, SP	Intestinal neuronal inflammation	[21,22]

NF-κB: Nuclear factor-kappa B; IL-1: Interleukin 1; MMPs: Matrix metalloproteinases; TNF: Tumor necrosis factor; IFN: Interferon; Th1: T helper 1; NMDA: N-methyl-D-aspartic acid; SP: Substance P.

Table 2 Magnesium and inflammation

	Role	Ref.
NF-κB Pathway	Inhibition of NF-κB p65 phosphorylation and stabilization of IκB protein	[29,39]
NMDA receptors, SP	Low magnesium enhances calcium influx through NMDA receptors	[12,38]
Neutrophilic oxidative stress	Increased levels of superoxide anions and nitric oxide in magnesium deficiency	[12]
Gut microbiota	Low magnesium changes the composition and intestinal permeability	[43]

NF-κB: Nuclear factor-kappa B; SP: Substance P; NMDA: N-methyl-D-aspartic acid; IκB: Inhibitory kappa B.

significantly if antibodies against IL-12 and IFNγ (*Fontolizumab*) are used as a potential therapeutic option[17]; however, this was eclipsed when the administration of these antibodies (anti-IL-12 and anti-IFNγ) showed a limited improvement in cases with active CD[20]. Recently, it was suggested that CD mucosal lesions are not caused only by Th1 cytokines, instead there is a possibility that other cells and mediators are also involved rather than Th1 cells alone as summarized in Table 1[17,20]. Examining mucosa of a terminal ileum from a CD patient before the appearance of lesions showed a large population of Th1 and macrophages releasing IFNγ and TNF-α, respectively; while samples from well-formed lesions presented a relatively equal response from Th1 and another set of cells, Th17, with dominance of their cytokines, IFNγ and IL-17A[17].

As shown in Table 2, this finding suggested a different set of active cells and cytokines presented as the disease progresses from early to late stages. Active lesions in CD are produced after a cascade of steps starting from the antigen presenting cells in the intestine. These cells get activated by luminal antigens triggering the differentiation of naïve T cells into either Th1 cells by IL-12 release or Th-17 cells by IL-6, IL-23, and transforming growth factor beta[4,17,20]. IL-21 from Th-1 and IL-17 from Th-17 will stimulate the release of matrix-degrading proteases from stromal cells[17,20]. Also, IFNγ from Th-1 will further activate macrophages that produce IL-1β and TNF-α, which will further trigger the release of more proteases[20]. Th17 are not stable cells, and as the inflammation continues to progress, they convert to Th-1 releasing more IFNγ during formation of late mucosal lesions[19]. This explains the persistence of high IFNγ levels towards late CD stages[20]. Therefore, it becomes clear that early stages of CD are dominated by Th1 cells, while late stages are mixed in control between Th1 and Th17 (Table 1)[17].

Furthermore, the effects of the adaptive immune system extend to the enteric nervous system. Neuronal inflammation and damage is a well-documented problem in IBD generally and in CD specifically[21,22]. A suggested mechanism for this pathology is explained through high activity of NMDA receptors in enteric neurons (Table 2). Leading to elevated levels of intracellular calcium, as a result, substance P (SP) will be released from these cells acting as a pro-inflammatory mediator increasing release of other inflammatory mediators such as TNF-α, IL-1 and IL-6 from macrophages and neutrophils, which adds to the overall exaggerated immune response in CD[21,22].

SP is a tachykinin peptide that has a high affinity for neurokinin-1 (NK-1) receptors on macrophages, neutrophils and mast cells, and the role of SP as a pro-inflammatory agent was proved when a specific antagonist for NK-1 receptors blocked the release of pro-inflammatory cytokines decreasing inflammation and severity of DSS-induced colitis in rats[22]. On the other hand, release of TNF-α and IL-1β has a role in the re-innervation of smooth muscle of intestine damaged in CD; through activation of NF-κB they were able to increase glial cell line derived neurotrophic factor expression in smooth muscles of intestine, making these cytokines neurotrophic and neurotoxic ones at the same time in a sense[21].

In contrast, the innate immune also plays a role in the pathogenesis of CD. Most of the commensal microbes we have in our bodies are located in the intestine, and this intestinal-microbial interface provides a large surface area for innate and adaptive immune response activities[23]. Cells like DC, M cells, and intestinal epithelial cells have the ability to detect and respond to these microbes[23]. This ability is provided by their surface expression of toll-like receptors (TLR), as well as intracellular NOD-like receptors (NLR), which are molecules that sense microbes through recognition of their PAMP[23-26]. PAMP that stimulates TLRs include bacterial lipoproteins, peptidoglycans and most importantly lipopolysaccharides (LPS)[24]. As shown in several studies[24,26,27], overexpression of TLRs (especially TLR2 and TLR4) is frequently observed in patients with IBD, which contributes to the dysfunction of immune tolerance to gut normal flora.

TLR and NLR promote the activity of NF-κB pathway, a major signaling pathway regulating the immune response through cytokine production and cell survival[24,25,28]. NF-κB molecules are transcription factors

stimulating a group of genes responsible for immune, inflammatory and apoptosis processes[24-26]. NF-κB induces the expression of its repressor IκB that binds to NF-κB molecules preventing their nuclear translocation[25]. Once IκB is phosphorylated, NF-κB becomes free to translocate into nucleus inducing the expression of pro-inflammatory cytokines[25,29]. Significance of this pathway in the inflammatory process of CD was established by the use of triptolide, a potent anti-inflammatory and immunosuppressant extracted from Chinese herb Tripterygium wilfordii Hook F[24]. The study showed that triptolide has down regulated TLR2 and TLR4 expression as well as NF-κB nuclear translocation, resulting in reduction in levels of pro-inflammatory cytokines in CD[24]. This finding suggested triptolide as a possible immunomodulator option for CD, and at the same time showed the strong link between TLR/NF-κB/pro-inflammatory cytokines in CD dysregulated immune response[24].

NUTRITIONAL LOSS IN CD

The inflammation caused by both innate and adaptive immune system leads to progression of CD, causing the loss of a significant functional intestinal area due to villous atrophy, fistulae formation and bacterial overgrowth[30]. These changes in the intestinal structure lead to loss of a variety of proteins, lipids, sugars, as well as vitamins and minerals, which locates the body in a negative nutritional status[13,15]. Other factors contributing to this negative balance in CD include: anorexia, abdominal pain, fasting for different tests, and medications like sulfasalazine used to control the disease or surgeries leading to short bowel syndrome[14,31]. For the same mentioned reasons, magnesium loss is a frequent finding in patients with CD as a result of the imbalanced magnesium homeostasis[13].

MAGNESIUM HOMEOSTASIS

This is maintained by the cooperation between three organs: intestine, kidneys and bones[32]. In the intestine, distal parts of jejunum and ileum are the most common sites for magnesium uptake[12,32]. Approximately 80%-90% of dietary magnesium absorption is achieved *via* paracellular transport, which depends on the permeability of tight junctions[32]. In addition, low expression of claudin 1, 3, 4, 5 and 8 proteins in the jejenum and ileum enables the passage of magnesium ions[33]. This mechanism is passive, allowing a majority of magnesium absorption without energy cost[12,32]. The rest of dietary magnesium is absorbed by the active transcellular transport *via* TRPM6 and TRPM7[12,32]. The latter mechanism allows magnesium to be transported into the blood from intestine through cell membrane[12,32]. Once absorbed, magnesium is stored mainly in bone tissue but traces can be found in muscles, where it acts as a natural calcium antagonist to control muscle contraction[12,32]. Lastly, most of the magnesium excreted from the body is processed by the kidneys, where 90%-95% of filtered magnesium daily gets retrieved *via* passive and active transport mechanisms[12,32].

MAGNESIUM IS NO TRACE ELEMENT: IT IS AN ESSENTIAL GIANT MINERAL

Magnesium is an important mineral in the human body like calcium, potassium and sodium[34,35]. When it comes to physiology, it is truly a "chronic regulator" and a "forgotten electrolyte"[34]. Magnesium is a cofactor for over 300 enzymes catalyzing phosphorylation reactions; it creates the proper conformational changes on their active sites so they fit their specific substrates, which regulates about 30% of total body proteins functions[34]. Regulation of cell cycle and apoptosis is achieved through many magnesium-dependent kinases, adding more weight on the significance of this mineral[34]. Production of the most common second messengers like c-AMP and c-GMP for different signal transduction pathways have magnesium involved in their regulation as well[34]. It is involved in the transport of many other electrolytes including calcium, potassium and sodium through its role in sodium/potassium ATPase activity, which explains the "refractory" nature of their disturbances to conventional treatment if the level of magnesium is low[34,36].

ROLE OF MAGNESIUM IN INFLAMMATION

Several studies have shown the importance of magnesium in inflammation that linked its low levels to many medical conditions such as diabetes type 2[37] (Barbagallo, 2007 #168), obesity, metabolic syndrome, osteoporosis, and cardiovascular diseases (Table 2)[12,38]. Levels of many pro-inflammatory cytokines varies depending on magnesium balance in the body, and among these cytokines, TNF-α IL-1 and IL-6 have the strongest relation[12,29,38]. Also, levels of CRP, a well-studied inflammatory indicator of low-grade and chronic inflammation synthesized by the liver, vary with magnesium status changes as well[12,38]. Effects of magnesium on inflammatory responses and mediators are widely distributed; therefore, it will be discussed separately as follows: (1) Magnesium as an anti-proinflammatory cytokine. Inhibition of NF-κB activity and increasing levels of IκBα are the backbone for this function (Table 2)[29]. NF-κB pathway is stimulated widely in the human body to regulate inflammation, cancer fighting, and cell survival[29]. Expression of cytokines IL-6 and TNF-α is induced during inflammatory responses triggered by TLR and NLR, which stimulates a downstream pathway to translocate NF-κB into nucleus for pro-inflammatory cytokines production[29,39]. IκBα is unstable due to its amino acids composition that is rich in proline, threonine and serine, explaining the constitutive breakdown rate affecting it[29,40,41]. IκBα level in monocytes were tested before and after magnesium sulfate supplementation following stimulation of TLR by LPS and it showed that level of

IκBα is higher in presence of higher intracellular magnesium[29]. The reason was not related to increased IκBα expression and protein synthesis, rather it was increased stability of IκBα, as proved by the use of protein synthesis inhibitor before and after TLR stimulation[29]. On the other hand, phosphorylation of NF-κB p65 is essential for its translocation, as well as its transcriptional effect to induce cytokines production; it was shown to decreased following magnesium supplement in LPS-TLR stimulated monocytes[29]. At the same time, expression of IκBα was decreased in the presence of increased intracellular magnesium sulfate supporting the finding of decreased activation of NF-κB in high levels of cellular magnesium sulfate[29,39]. As a result of inhibition of NF-κB translocation and transcriptional effect and the decreased phosphorylation of NF-κB, levels of TNF-α and IL-6 were significantly lower in LPS-TLR stimulated cells in the presence of high cellular magnesium levels[29,39]. Also, magnesium has preserved and stabilized more IκBα, which led to more suppression in NF-κB related cytokine production following LPS stimulation. In another study, it was shown that IL-8 expression is decreased following the same steps mentioned for LPS stimulation of cytokine production[39]; (2) Oxidative stress and magnesium. In an experiment conducted on magnesium deficient rats, there was a 40% increase in the level of superoxide anions and nitric oxide levels (Table 2)[12]. Also, there were increased levels of neutrophilic basal superoxide anions, as well as prostacyclin, prostaglandin E2, and thromboxane A2[12]. Red blood cells glutathione levels were decreased in the same experiment showing declining body antioxidant potentials in increased oxidative stress as a result of low cellular magnesium levels[12]; (3) Magnesium effect on NMDA receptors. Magnesium is a natural calcium antagonist and this was discussed in role of magnesium in muscle contraction[32]. From another perspective, NMDA receptors have a threshold of activation and it is lowered in states of decreased extracellular magnesium levels[38]. This will lead to an increase in calcium influx into the cell through NMDA receptors, resulting in increased production of pro-inflammatory prostaglandin E2, which was decreased upon blocking NMDA receptors[12,38]. Also, as calcium levels increases intracellular, the level of SP increases as a result stimulating NK-1 receptors leading to production of inflammatory mediators from macrophages, monocytes and neutrophils[38]. It is noteworthy to mention that the increase in NK-1 and substance P are well-known findings in IBD[12]. In addition to that, magnesium binds to the regulatory gates of calcium channels limiting calcium influx into the cell, and low extracellular magnesium levels will enhance the calcium influx triggering a greater inflammatory response[38]; (4) Magnesium, gut microbiota and intestinal permeability. It has been established before that gut microbiota [mainly bifidobacteria; a gram positive, non-motile anaerobic bacteria (Table 2)[42]] are decreased in endotoxemia, high fat mass index and glucose utilization disturbances[43,44]. Similarly, in another experiment, cecal content of bifidobacteria and lactobacilli were decreased in short-term (four days) magnesium deficient rats[43]. On the other hand, prolonged magnesium deficiency (21 d) has actually increased the cecal content of the mentioned bacteria, suggesting an adaptive response by the bacteria and an established demand for magnesium[43]. Bifidobacteria are microorganisms known for their ability to lower intestinal LPS content and thus enhance the mucosal barrier performance[43,45]. As the drop of magnesium levels decreases the cecal bacterial content, it also causes change in intestinal mucosal barrier, where mRNA of two of the junction proteins (ZO-1 and Occ) were noticed to decrease in ileum and proximal colon resulting in increased intestinal permeability for bacterial products and especially LPS to be increased systemically[43]. Accordingly, it was noticed that expression of CD14 receptors that bind LPS was elevated in gut in magnesium deficient mice, as well as increased expression of CD68 supporting the infiltration of monocytes in proximal colon[43]. The overall content of mRNA of TNF-α and IL-6 in proximal colon was increased in magnesium deficient mice[43]. These findings showed the effect of low magnesium on cellular inflammatory stress, which seemed to be limited to proximal colon rather than ileum[43]. Prolonged magnesium deficiency has an impact on the composition of gut microbiota as more bifidobacteria and lactobacilli will be present, and less bacteroids in the intestine[43]; and (5) Magnesium and C-reactive protein. As several studies investigated effects of dietary modification on inflammatory processes, CRP was among the most common inflammation indicators used for evaluation[46]. High levels of CRP were linked to obesity, metabolic syndrome, cardiovascular diseases and IBD[47]. They all share having an inflammatory component in their etiology and CRP was the tested variable in many studies[12]. As levels of IL-1β, IL-6 and TNF-α increase in the plasma, liver will respond by increasing production of CRP[38]. More specifically, serum high sensitive CRP (hs-CRP) has been used frequently due to its stability and easy detection, which has a normal level in plasma of < 3.0 mg/L[48]. Different conditions with low-grade or chronic inflammation states shared the sign of having elevated hs-CRP, indicating the strong inflammatory component they have[46]. CD activity has been strongly correlated to hs-CRP level[49], which is considered to be one of the main laboratory values that increase in relapses. Back to our mineral, magnesium is a significant immunomodulator that affects many inflammatory responses, and therefore its homeostasis is crucial for the overall body homeostasis. Low magnesium levels (< 1.2 mg/dL) were correlated to elevated levels of TNF-α, IL-1β, IL-6 and hs-CRP in plasma[12,38,46]. A study conducted on 5007 children (1999-2002) showed a significant increase in risk of having high CRP (1.94 times more) in children taking less than 50% of magnesium RDI[38]. One of the most interesting findings about magnesium and hs-CRP is that it was developed at University of South Carolina, showing that magnesium is the highest dietary factor in a 42-item dietary anti-inflammatory index they made for the study[46,48].

MAGNESIUM LOSS IN CD

With more than 32% of American people not meeting the daily requirement of magnesium dietary intake (4.5 mg/kg per day for adults), hypomagnesemia became a real concern for many practitioners[14]. Therefore, IBD adds a major cause for developing hypomagnesemia at different rates ranging from 13% to 88% of patients[14]. This deficiency is caused by many factors in CD including anorexia, food avoidance, intestinal surface loss due to diarrhea, fistulae or surgery as well as malabsorption[14]. Intestinal uptake of magnesium is defected dramatically as inflammatory processes of CD result in villus atrophy and fistulae formation, on top of increased bowel movement not allowing the time for magnesium absorption[32]. As the majority of magnesium absorption occurs passively, there will be no sufficient concentration gradient for magnesium uptake in intestine, as well as destructed enterocytes, losing the active transport component of magnesium absorption[32].

Nutritional loss in patients with CD is variable based on the disease activity status. Usually during remission of the disease, the body demand for macronutrient is covered by diet. However, micronutrient loss is frequent and supplementation is usually required even during remission of the disease[15]. Due to the chronic and extensive damage of intestinal mucosal cells, oral magnesium supplement is not recommended and parenteral forms are encouraged since the bioavailability will not be a concern in this case[13].

As CD result in malabsorption and loss of many vitamins, vitamin D in particular has a direct influence on magnesium and its intestinal absorption[32]. Claudin proteins involved in paracellular mechanism of magnesium absorption (the major mechanism) are regulated by active vitamin D, thus in CD, loss of fat soluble vitamins including vitamin D will lead to decreased magnesium absorption and hypomagnesemia[32]. As 75% of CD patients will require surgery at some point due to intestinal disease complications, short bowel syndrome will be a major cause of malabsorption affecting the levels of many nutrients including magnesium as well[14].

Also, magnesium absorption in the intestine is subjected to the amount of protein in diet and this is decreased in CD due to anorexia produced by circulating cytokines and food avoidance by the patients because of abdominal pain[32]. For all of those factors, magnesium will be in negative balance in CD patients (Figure 1).

EFFECTS OF MAGNESIUM ON CROHN'S PATHOGENESIS

By looking back at magnesium significance in the human body, it is obvious that the major effect magnesium can have on CD is from the immunity and inflammation point of views. However, other fields for magnesium influence on CD can be calcium disturbances, intestinal nerve supply and gut microbiota composition.

As established before in this paper, magnesium has the potential to be an effective cytokine antagonist. In different studies magnesium showed immunomodulation capabilities through controlling expression of pro-inflammatory cytokines, oxidative stress and neuronal damage. Through its effect on NF-κB, intracellular magnesium was able to limit NF-κB nuclear translocation as well as p65 phosphorylation activation step. On the other hand, intracellular magnesium preserved and stabilized IκBα limiting its degradation and applying more inhibitory effect on NF-κB pathways. As CD progresses and intestinal lesions develop, exposure of TLR and NLR to LPS will be more likely (Table 2). LPS is a potent TLR stimulator, which will activate NF-κB subsequently leading to production of IL-1, IL-6 and TNF-α. Hypomagnesemia is a frequent finding in CD, which means that the inhibitory effect on NF-κB pathway will be absent allowing more production of IL-6 and TNF-α that will trigger more mucosal damage *via* activation of the release of matrix-degrading proteases from intestinal stromal cells[20].

Among the structural changes that occur to intestinal mucosa due to CD, gut microbiota composition changes are also related to magnesium deprivation[43]. Short-term and long-term magnesium deficiency in mice showed significant changes in intestinal permeability and bacterial adaptation[43]. Low magnesium levels decreased bifidobacteria and increased risk of LPS endotoxemia since bifidobacteria can lower intestinal content of LPS[43]. Down-regulation of junction proteins ZO-1 and Occ mRNA as a result of magnesium deprivation caused an increase in intestinal permeability and this finding alone can describe the significant effect of hypomagnesemia in CD patients[43]. Role of magnesium in alleviating LPS immune response is essential for immune tolerance to gut commensal bacteria.

Among the inflammatory mediators associated with CD, CRP is one of the most sensitive markers of CD activity and relapsing status[50]. Its short half-life made it superior to other markers like ESR and fibrinogen which have longer half-lives and interference with other agents[50]. As demonstrated in many studies[12,38], serum CRP levels are elevated in diabetes type 2, obesity, metabolic syndrome, atherosclerosis, osteoporosis and alcoholism indicating the inflammatory component that links them together in etiology. At the same time, all of these conditions were also associated by hypomagnesemia suggesting a strong relationship between magnesium and CRP and its possible application on CD where levels of magnesium are reduced[12,38].

Calcium influx into neurons is largely regulated by extracellular magnesium[12,38]. Low magnesium levels due to CD will lower NMDA receptors activation threshold allowing more calcium entry into neurons[12,38]. Also, gated calcium channels will lose magnesium regulation over them and will allow more calcium influx, which adds to the overall free intracellular calcium[12,38]. Elevated levels of intracellular calcium are able to increase the release of

inflammatory SP that trigger more production of IL-1, IL-6 and TNF-α resulting in increase in the oxidative stress affecting intestinal sensory innervation causing symptoms like tenesmus (feeling of incomplete defecation) and frequent bowel movements[12].

Hypocalcaemia in CD is characteristic and has more than one cause resulting loss of many calcium functions throughout the body. First, as more vitamin D is lost in the frequent diarrhea associated with CD, calcium absorption at the intestine will be impaired[32]. On the other hand, low magnesium levels associated with CD results in increased calcium influx shifting most calcium into cells and causing calcium levels in plasma to drop even more[12,38].

Role of magnesium in muscle contraction as a calcium antagonist is essential for intestinal smooth muscle function in creating efficient peristalsis. It allows for periods of relaxation following contraction cycles caused by calcium. Also, SP is a regulator of smooth muscle contractility and hypomagnesemia elevates its levels leading to abnormal intestinal smooth muscle function. This function is essential to control bowel movement frequency in CD which is increased as a result of magnesium deficiency[32].

As levels of antioxidant vitamins like vitamin A, vitamin C and vitamin E are decreased in CD due to intestinal loss, oxidative stress effect on different cells increases including intestinal cells as well. Low magnesium levels showed an association with increased oxidative stress in individuals with no CD as levels of lipid peroxidation increase and production of free radicals like superoxide anion and nitric oxide is promoted[12]. These changes in CD with addition of hypomagnesemia augment the load of oxidative stress all over the body.

TARGETS FOR FUTURE DIRECTIONS

The integration of magnesium functions throughout the body is enormous and fields of future studies of that are numerous. However, when it comes to CD association with hypomagnesemia, some targets are very promising for possible maintenance of remission or even a cure of CD.

Most of drugs used to control CD have a long list of side effects and a possible toxicity with chronic use. Magnesium has shown great potentials on affecting the same pathways involved in CD inflammation as many therapeutic agents. On the other hand, magnesium is not expected to be cytotoxic and this hypothesis is very promising if magnesium is tested on specific regimens to block NF-κB signaling pathway in CD patients.

Role of magnesium in controlling calcium entry in neurons is significant in intestinal sensory innervation[12]. Hypomagnesemia leads to more nerve damage following SP activation and this effect carries a potential of modifying intestinal smooth muscle function and innervation in CD if magnesium supplement are tested for that.

Another area that is poorly understood is the role of magnesium in CD as a cofactor for most kinases and the possible changes of this rule during the disease. It is unclear whether this function affects CD activity and if so, in what way this is applied in cases of hypomagnesemia associated with CD.

Possible changes in gut microbiota composition following magnesium level alterations represent a big opportunity to further explore the role of normal flora in developing CD. Other minerals could play a similar role for the short term or long term changes in their levels. As data suggested, certain strains of microbiota like bifidobacteria turned out to have a role in lowering LPS and contributing to the intestinal mucosal barrier function and tolerance. Could other strains have different functions involved in immune responses and tolerance? A hypothesis can be based on the most common commensal strains and their possible role in local and systemic immune responses and possible implications in autoimmune diseases.

Magnesium supplementation for CD patients is strongly suggested by several research data. Maintaining magnesium homeostasis throughout the course of the disease is expected to minimize the inflammatory damage of CD improving the condition of many patients. However, conventional magnesium supplementation itself causes diarrhea which is the main reason magnesium is lost in CD. A therapeutic strategy for magnesium administration is strongly recommended.

CONCLUSION

CD is primarily an innate immunity dysfunction, and this disturbance develops to trigger an adaptive immune response resulting in mucosal intestinal surface damage. Among the numerous functions magnesium has throughout the body, immunomodulation is by the far the most involved function in CD activity and development. Chronic diarrhea among other problems in CD results in long term loss of magnesium, which makes hypomagnesemia a frequent finding in most CD patients. As the data showed, restoration of magnesium levels in CD patients can limit the activity of NF-κB, which is responsible for production of pro-inflammatory cytokines involved in CD inflammation. Additionally, many studies have suggested the disturbances in gut normal flora composition following short and long term hypomagnesemia. These have resulted in loss of immune tolerance to normal flora at the intestinal interface. Involvement of hypomagnesemia in NMDA receptors and SP release creates a direct effect on neuronal function of intestine and smooth muscle activity as well.

This review highlights some of the well-known functions of magnesium and their potential rule in shaping CD activity. It was strongly suggested by data that magnesium could play a significant rule in controlling CD. This review demonstrates a possible mutual effect of CD on magnesium level as well as hypomagnesemia on CD inflammatory processes. Future nutritional studies as well

as medical research are expected to focus more efforts to better understand effects of magnesium and CD on each other. Knowledge of these effects would create a strong basis for development of a potential therapeutic strategy to modulate the vast inflammatory effects CD has on its patients.

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P- Reviewer: Izawa KP, Zhao JB **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Choice of wound care in diabetic foot ulcer: A practical approach

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Received: November 11, 2013 Revised: May 23, 2014
Accepted: May 29, 2014
Published online: August 15, 2014

Abstract

Diabetic foot ulcers are the consequence of multiple factors including peripheral neuropathy, decreased blood supply, high plantar pressures, *etc.*, and pose a significant risk for morbidity, limb loss and mortality. The critical aspects of the wound healing mechanism and host physiological status in patients with diabetes necessitate the selection of an appropriate treatment strategy based on the complexity and type of wound. In addition to systemic antibiotics and surgical intervention, wound care is considered to be an important component of diabetic foot ulcer management. This article will focus on the use of different wound care materials in diabetic foot. From a clinical perspective, it is important to decide on the wound care material depending on the type and grade of the ulcer. This article will also provide clinicians with a simple approach to the choice of wound care materials in diabetic foot ulcer.

Key words: Diabetes; Foot; Wound; Debridement; Topical

Core tip: Diabetic foot ulcers are an important complication of diabetes. There is no conventional guideline regarding the selection of wound care materials in diabetic foot wounds. This article includes fundamental aspects of wound care and management with special emphasis on the selection of appropriate wound care materials depending on the type of wound tissue. Risk factors for foot ulceration, classification and grading of wounds, bacteriology, multidisciplinary team approach, types of debridement, importance of offloading, wound care and choice based on the complexity of the wound and properties of the dressing regime in each category based on clinical experience and practice are discussed.

Original sources: Kavitha KV, Tiwari S, Purandare VB, Khedkar S, Bhosale SS, Unnikrishnan AG. Choice of wound care in diabetic foot ulcer: A practical approach. *World J Diabetes* 2014; 5(4): 546-556 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/546.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.546

INTRODUCTION

The increasing prevalence of diabetes has resulted in concomitant illness[1]. The critical effects of hyperglycemia include micro-vascular complications (nephropathy, neuropathy and retinopathy) and macro-vascular complications (coronary artery disease, stroke and peripheral arterial disease). Diabetes is a leading cause of non-traumatic lower extremity amputation, which is often

Figure 1 Wound classification based on the Red-Yellow-Black wound classification system by Marion Laboratories. A: Necrotic tissue; B: Sloughy tissue; C: Granulating tissue; D: Epithelializing tissue.

preceded by a non-healing ulcer. The lifetime risk of foot ulceration in people with diabetes is 15%-20%[2]. More than 15% of foot ulcers result in amputation of the foot or limb[3]. Several other population-based studies indicate a 0.5%-3% annual collective incidence of diabetic foot ulcers. The prevalence of foot ulcers reported varies from 2% to 10%[4]. Approximately 45%-60% of all diabetic foot ulcerations are purely neuropathic, whereas 45% have both neuropathic and ischemic components[5]. It has been estimated that around 15%-27% patients with diabetes require lower limb amputations predominantly (50%) due to infection[6].

DIABETIC FOOT

Definition

Infection, ulceration or destruction of deep tissues associated with neurological abnormalities and various degrees of peripheral vascular diseases in the lower limb (World Health Organization definition, 1995).

Risk factors

Diabetic foot ulcers are a consequence of many factors including loss of protective sensation due to peripheral neuropathy where the feet become numb and the injury goes unnoticed. Also, arterial insufficiency complicates the neuropathic ulcer which leads to poor wound healing. Foot deformity and calluses can result in high plantar pressure, which results in additional risk. Mechanical stress at the wound site is hypothesized to affect wound healing[7]. Many other factors contribute to the risk of foot ulceration and its subsequent infection in patients with diabetes. Uncontrolled hyperglycemia, duration of diabetes, trauma, improper footwear, callus, history of prior ulcers/amputations, older age, blindness/impaired vision, chronic renal disease and poor nutrition have also been demonstrated to play a role in the pathogenesis and progression of diabetic foot ulceration. Infection further deteriorates the diabetic foot resulting in a non-healing chronic wound. Recently, vitamin D deficiency was proposed as a risk factor for diabetic foot infection[8].

Classification

Based on the Red-Yellow-Black[9] wound classification system by Marion Laboratories, wounds can be classified as follows[10]: (1) Necrotic tissue-either dry or infected and usually black or dark green in color as shown in Figure 1A; (2) Sloughy tissue-combination of wound exudate and debris forming a glutinous yellow layer of tissue over the wound which is often mistaken for infection as shown in Figure 1B; (3) Granulating tissue-highly vascularized, red in color and sometimes highly exudating as shown in Figure 1C; and (4) Epithelializing tissue-Epithelium grows over a wound formed by migration of keratinocytes from the wound margins, which looks pink in color as shown in Figure 1D.

Debridement of necrotic tissue is an integral component in the treatment of chronic wounds as they do not heal in the presence of unviable tissue, debris, or critical colonization[11,12] and may be contraindicated in arterial ulcers[13]. Excision of necrotic tissue is necessary for wound healing. Calluses or thickened skin surrounding the ulcer

need to be excised. Necrotic tissue removed on a regular basis can expedite the rate at which a wound heals and has been shown to increase the probability of attaining full secondary closure[14,15].

Grading

Grading can be done using Wagner's or the Texas wound classification system[16]. The most common is the University of Texas wound classification system, which describes the wound with regard to depth, presence or absence of infection or ischemia or both. A description of the wound is important for wound care choice and includes the location, stage, dimension in length, breadth and depth (length and breadth can be measured in centimeters by tracing it on a sterile acetate sheet and depth can be taken by inserting a sterile swab gently into the deepest part of the wound), wound edges (undermining), wound base description, drainage (heavy or low), color, odor, pain and progression, *etc*[17].

Microbiology

Hyperglycemia, impaired immunologic responses, neuropathy, and peripheral arterial disease are the major predisposing factors leading to limb-threatening diabetic foot infections[18-20]. The prevalence of infection in India was 6%-11%, whereas the prevalence of amputation was 3% in patients with type 2 diabetes[21]. Both aerobic and anaerobic bacteria have been shown to infect diabetic foot wounds[22-25]. Fungal infections are also common in diabetic foot[26-28]. Polymicrobial etiology of diabetic foot infections has been widely reported[22-25,29]. However it is not uncommon to have a predominance of mono-microbial infection in diabetic foot[30]. Researchers have shown the predominance of both gram negative[30] and gram positive[26] bacteria in diabetic foot infections. Various studies have reported a high prevalence of *Pseudomonas*[31], *E. coli*[30], and *S. aureus*[26,30] infections in diabetic foot. The pattern of microbial infection in patients with diabetic foot infections is inconsistent and therefore evaluation of microbial characteristics and their antibiotic sensitivity is necessary for the selection of appropriate antibiotics for management of diabetic foot infection.

MANAGEMENT TECHNIQUES

The foot is a complex structure, which acts as a foundation for the whole body, and it is important to prevent progression of diabetic foot problems. The integration of knowledge and experience through a multidisciplinary team approach promotes more effective treatment, thereby improving outcomes and limiting the risk of lower extremity amputation[32,33]. Therefore the following specialists play an important role: (1) Endocrinologist/Diabetologist (optimize blood glucose control); (2) Podiatrist (focus on the foot including prevention and treatment of diabetic foot wounds); (3) Vascular surgeon (manage vascular issues); (4) Microbiologist (look into microbiological etiology and antibiotic selection based on cultures); (5) Orthotist (ensures that therapeutic or custom made footwear aids in minimizing pressure); and (6) Nutritionist (concentrates on diet which helps in the management of diabetes as well as wound healing).

Wound healing is a complex process involving highly regulated responses of specified cell types, which harbor locally secreted growth factors that play a key role in wound healing[34]. Treating a diabetic foot infection requires proper wound care and appropriate antibiotic therapy[19]. The fundamentals of good clinical care includes adequate frequent debridement, offloading, moist wound care, treatment of infection, and revascularization of the ischemic limb[35]. In addition, wound healing can be enhanced by the appropriate choice of a topical regime (mixed range of standard and advanced topical therapies), however, adequate training and significant clinical experience are essential for making this choice. Many factors including assessment of the wound, its classification, and the need for debridement including sharp surgical, mechanical, chemical, *etc*., have to be taken into consideration before proceeding with the appropriate selection of topical regimen.

Debridement

Debridement involves removal of dead, damaged, or infected tissue, which improves the healing potential of the remaining healthy tissues. Depending on the wound tissue type, different debridement techniques are recommended[36,37]: (1) Surgical debridement or sharp debridement-recommended for necrotic and infected wounds. The terms surgical debridement and sharp debridement are often used synonymously, some clinicians refer to surgical debridement as being performed in an operating room, whereas sharp debridement is performed in a clinic setting[38]. Sharp surgical debridement is the most effective and fastest method of debridement; (2) Autolytic debridement-a selective process in which the necrotic tissue is liquefied. A wound covered with an occlusive dressing allows accumulation of tissue fluids containing macrophages, neutrophils, and enzymes, which remove bacteria and digest necrotic tissues. This is achieved by a moist wound healing environment[36]. Autolytic debridement is not advisable for the treatment of infected pressure ulcers[39]; (3) Mechanical debridement-involves removal of unhealthy tissue using a dressing, which is changed regularly by wound irrigation (pressure: 4-15 psi), without damaging healthy/new tissues[40]. Scrubbing the wound aids in removal of exudates and devitalized tissues, however this leads to bleeding as well as pain resulting from wound trauma. This technique is used in the management of surgical wounds and venous leg ulcers. The drawbacks of the method is that it is time consuming and expensive; (4) Enzymatic debridement-a method of debriding devitalized tissue by topical enzymes such as collagenase, fibrinolysin, or papain. Recommended for sloughy, infected, necrotic wounds where surgical debridement is contraindicated[41]; and (5) Maggot debridement-a technique in which maggots or fly larva that

Table 1 Antibiotic recommendation based on the severity of the infection

Site	Severity or extent	Route of administration	Duration of therapy
Soft tissue only	Mild	Topical or oral	1-2 wk may extend up to 4 wk if slow to resolve (outpatient)
	Moderate	Oral (or initial parenteral)	1-3 wk (Outpatient/inpatient)
	Severe	Initial parenteral, switch to oral when possible	2-4 wk (Inpatient, then outpatient)
Bone or joint	No residual infected tissue (*e.g.*, post-amputation)	Parenteral or oral	2-5 d
	Residual infected soft tissue (but not bone)	Parenteral or oral	1-3 wk
	Residual infected (but viable) bone	Initial parenteral, then consider switching to oral	4-6 wk
	No surgery, or residual dead bone post-operatively	Initial parenteral, then consider switching to oral	⩾ 3 mo

are raised in a sterile environment are used. The most commonly used fly is Lucilia sericata, which is used for human wound treatment when conventional treatments fail[42]. Maggots are placed on the wound followed by wrapping with secondary dressing. The larvae feed on the necrotic (dead) tissue and bacteria present at the wound site and secrete antimicrobial enzymes, which help in the wound healing process.

Offloading

Completely or partially relieving pressure from the weight bearing area of the foot by providing mechanical support with the intention of giving rest to the wound area aids in healing. Repetitive trauma and high plantar pressure on the ulcer bed are two primary reasons for the persistence of ulcers once they have developed[43]. Offloading is very important in diabetic wound healing. There are many types of offloading techniques including total contact casts, removable cast footwear, wedge footwear, half shoes, mobilization by wheelchair, *etc*. Total contact casts are considered to be the gold standard method of offloading and treating diabetic patients with neuropathic ulcers[32,44-46].

Wound care

Wound care plays a pivotal role in the management of diabetic foot ulcer, which comprises cleaning the wound with normal saline following aseptic techniques and the use of modern wound care techniques that promote a moist wound healing environment[47,48]. Although topical treatment is an important aspect of wound care, it is always considered secondary to surgical and systemic care[49]. There are numerous topical regimens and devices available for the management of diabetic foot wounds including hydrogels, hydrocolloids, alginates, foam, silver impregnated dressings, growth factors, silicon impregnated atraumatic dressings, vacuum aided devices, hyperbaric oxygen therapy, *etc*. However, before choosing a regime one should consider factors such as the general health of the patient, the process of tissue repair, assessment of the wound by means of grading, description and classification of the wound, local environment of the wound, knowledge on specific properties of the dressing materials and devices as well as their availability, affordability, and accessibility.

The ideal characteristics of a wound dressing are as follows[50,51]: (1) Sterile, easy to use, cost effective; (2) Maintain a moist wound healing environment; (3) Absorb excess exudate; (4) Non-adherent/non-toxic, non-allergic; (5) Not contaminate the wound with foreign particles; (6) Protect the wound from microorganisms; (7) Allow gaseous exchange and control wound odor; and (8) Provide thermal insulation and mechanical protection.

Antibiotic selection

The principle of antibiotic treatment is based on evidence provided by reports on bacteriological culture and sensitivity from different centers worldwide[52,53].

Use of anti-infectives/antibiotics must be guided by appropriate cultures. Inappropriate use of antibiotics could lead to resistance and adverse effects.

Oral and parenteral antibiotics are prescribed for mild soft tissue infections and moderate to severe infections, respectively (Table 1)[54]. Evidence-based regimes should be followed for the management of infection in diabetic foot. Appropriate dosage, optimal duration, identification and removal of the infective focus and recognition of adverse effects should be critically evaluated in all outpatients and inpatients with diabetic foot infections[54-56].

Every hospital should develop an institutional antibiotic policy containing guidelines and protocols for antibiotic use. It is advisable to have different sections for treatment and prophylaxis including surgical procedures as well as how to treat different infections[57].

Three levels of antibiotic prescribing are generally recommended: (1) First line of choice - antibiotics prescribed by all doctors; (2) Restricted antibiotic group - for resistant pathogens, polymicrobial infections, special conditions, and expensive antibiotics. When prescribing antibiotics from this group, the prescriber should discuss with the committee and head of the department; and (3) Reserve antibiotics-for life-threatening infections, to be used after obtaining permission from the committee.

The institutional antibiotic committee should update their policy by collecting surveillance on antimicrobial resistance and data on antibiotic consumption, which will improve clinical and laboratory standards. The committee should monitor implementation of the policy,

Table 2 Choice of wound care materials for necrotic and sloughy wounds

Wound classification	Choice of wound care material	Advantages	Disadvantages
Necrotic wound	Wet to dry	Good debriding capacity and inexpensive	Frequent dressing change Painful if not soaked with saline prior to dressing change
	Topical antibacterial such as metronidazole	Very good antibacterial coverage Maintains a moist wound healing environment by promoting autolysis and controls odor	Chance of maceration Contraindicated in infected necrotic wounds
	Hydrogel	Hydrates the wound by promoting autolysis	Chance of maceration Contraindicated in infected necrotic wounds and is expensive
	Hydrocolloid	Maintains a moist wound healing environment, which helps in autolytic debridement	Expensive Contraindicated in infected necrotic wounds
Sloughy wound	Wet to dry	Good debriding capacity Absorptive, adhesive and cheapest	Frequent dressing change Painful if not soaked with saline prior to dressing change
	Topical enzymes such as collagenase, papain, fibrinolysis	Promotes autolytic debridement by desloughing Can be used in combination with metronidazole or hydrogel	Contraindicated in granulating or epithelizing wounds
	Topical antibiotics such as metronidazole	Very good antibacterial coverage Maintains moist wound healing environment by promoting autolysis and controls odor	Chance of maceration
	Polyurethane Foam	Very effective in desloughing Maintains a moist wound healing environment by promoting granulation	Sometimes painful if not soaked with saline prior to dressing change
	Hydrogel	Hydrates the wound by promoting autolysis	Chance of maceration and is expensive
	Hydrocolloid	Maintains a moist wound environment, which helps in autolytic debridement	Chance of maceration and is expensive

receive feedback information, assess the outcome, and discuss with various specialty doctors. The policy should be reviewed every year based on the experience of prescribers and the susceptibility reports of microbiology and laboratory.

Revascularization

With advances in both vascular and orthopedic reconstructive surgeries, limb salvage has become an option for limbs that previously would have been amputated. Patients with both diabetes and peripheral arterial disease are more prone to ischemic ulceration than those without the disease[58,59]. Several endovascular options, including percutaneous transluminal angioplasty (PTA), balloon-expandable stents, self-expanding stents, and covered stents are now available. The success rate after stent implantation in the iliac arteries is greater than 95%[60]. Revascularization plays a crucial role in the treatment of ischemic lower extremity wounds and should be performed before drainage or debridement[61]. Endovascular techniques such as cryoplasty, drug eluting stenting, plaque debulking lasers, *etc*., are being investigated and are potentially useful adjuncts to PTA. Subintimal angioplasty for arterial lesions below the ankle in diabetic patients could achieve a limb salvage rate of 94.6%[62]. Several retrospective studies report considerably better results of transmetatarsal amputations performed after a revascularization procedure[63,64].

CHOICE OF TOPICAL REGIME

Choice of wound care materials should be based on wound tissue type, complexity, and its properties (Tables 2 and 3).

Wet to dry dressing or simple saline

This dressing has a good debriding action and helps in wound bed preparation. Wet-to-dry dressings are described in the literature as a means of mechanical debridement[65]. It is very absorptive as well as adherent and one of the cheapest dressings used throughout the world, but requires frequent dressing change (twice or thrice a day) based on wound severity. Dressings should be moistened before removal to minimize any chance of bleeding. A gentle cleanser (normal saline or neutral-pH cleanser) will minimize wound irritation and discomfort[66]. When treating a granulating or epithelizing wound one should soak the dressing thoroughly with normal saline for five minutes (based on our clinical experience) to prevent trauma and heavy bleeding.

Antibacterial agents

Used solo or in combination for each category except dry necrotic wounds. Topical antibiotics have broad-spectrum antibacterial coverage which lasts for 12 h and are less toxic. Metronidazole gel [Ornidazole (IP-10 mg and water soluble gel base quantity sufficient)] has good anaerobic coverage and helps in maintaining a moist wound healing environment. By weight, gels are mostly liquid, yet they behave like solids due to a three-dimensional cross-linked network within the liquid. It is the crosslinking within the fluid that gives a gel its structure (hardness) and contributes to its adhesion[67]. Both by

Table 3 Choice of wound care materials for healing/sinus or cavity wounds

Wound classification	Choice of wound care materials	Advantages	Disadvantages
Granulating wounds	Non adherent dressing	Reduces trauma to the healing tissue Maintains a moist wound healing environment	Chance of shearing to new epithelium
	Wet to dry dressing	Promotes healing	Chance of bleeding if not soaked with saline before dressing change
	Polyurethane foam	Maintains a moist wound healing environment Promotes healing process	Chance of bleeding if not soaked before dressing change
	Topical antibacterial such as metronidazole, mupirocin, Tulle, Silver containing ointments, Acetic acid 0.5%-5% and povidone iodine	Maintains a moist wound healing environment, promotes epithelization and controls odor Effective against Gram positive cocci including MRSA. Silver sulfadiazine has broad antibacterial coverage, accelerates epithelization, and is very effective in burns. Acetic acid is very effective against Pseudomonas. Povidone iodine is very effective for gangrene as it hastens demarcation	Silver containing ointments cannot be used in Sulfa allergy patients Povidone iodine is cytotoxic to fibroblasts and delays the healing process
	Platelet derived growth factor	Faster healing and very effective	Expensive
	Hydrogel	Promotes healing	Chance of maceration and is expensive
	Hydrocolloid	Promotes healing Reduces the interval of dressing change	Chance of maceration and is expensive
Epithelizing wounds	Non adherent	Reduces trauma to the healing tissue Maintains a moist wound healing environment	Chances of shearing
	Wet to dry dressing	Promotes faster healing	Soaking of dressing is required prior to dressing change
	Topical antibacterial	As mentioned in granulating wounds	As mentioned in granulating wounds
	Epidermal growth factor	Effective and faster healing	Expensive
	Hydrogel	Effective	Chance of maceration and is expensive
	Hydrocolloid	Effective	Chance of maceration and is expensive
Cavity/Sinus wounds	Alginate	Highly absorbent and non-adherent Maintains a moist wound healing environment	Needs adequate padding and is expensive
	Hydrogel	Effective in promoting granulation tissue	Needs adequate padding and is expensive

weight and volume, gels are mostly fluid in composition and thus exhibit densities similar to those of their constituent liquids, such as hydrogels. Topical metronidazole gel (0.75%-0.80%) is frequently used directly on the wound once per day for five to seven days or more often as needed[68,69], and metronidazole tablets can be crushed and placed onto the ulcer bed[66,70]. There are numerous other articles (case studies or anecdotal experience) reporting the reduction of wound odor with topically applied metronidazole[71-73]. Antibiotics such as Neomycin, Gentamycin, and Mupirocin have good antibacterial coverage when used topically. Silver containing dressings come in different formulations and have very good antibacterial coverage. Silver dressings and polyherbal preparations have shown good results in healing diabetic foot wounds[74]. They are very effective in burn wounds and can also be used in infected or colonized wounds. Sisomycin (0.10%) and acetic acid at concentrations between 0.5% and 5% are effective against *Pseudomonas*, other gram-negative bacilli, and beta hemolytic streptococci wound infections. Povidone iodine solution dressings are very effective in healing sutured wounds and hypergranulating wounds to suppress or hamper further granulation. Povidone iodine soaked gauze is a good dressing for dry gangrene which hastens the process of demarcation. Iodine has been found to be toxic to human cells as well as bacteria and fungi at high doses[75,76]. Also, it should not be used on granulating or epithelizing wounds because it slows down the healing process and is cytotoxic to keratinocytes and fibroblasts.

Tulle dressings

These are gauze dressings impregnated with paraffin, which lowers the dressing adherence, but this property is lost if the dressing dries out. Tulle dressings are mainly indicated for superficial clean wounds and skin grafts. They can be used in granulating and epithelizing wounds. Tulle dressings not only prevent trauma to the new and delicate epithelium during dressing removal, but also provide a good moist environment, which is preferred for epithelial cell proliferation and migration[77]. This concept is well supported by evidence from many previous studies which showed faster re-epithelialization rates when moist environment dressings were compared with traditional dry dressings[77-79]. Evidence shows that gauze-based dressings still have a place in wound care[80].

Polyurethane films

These films are coated with an adhesive (water-proof dressing) and are comfortable. The vapor-permeable films allow diffusion of gases and water vapor which helps in maintaining a moist wound healing environment. Their transparency allows for wound monitoring without dressing removal, but there is a chance of maceration of surrounding skin. They can be used for low exudating wounds.

Polyurethane foam

These dressings are extremely absorbent, non-adherent, and have a semi-permeable backing which allows moisture to escape. Polyurethane foam dressings loosen slough by creating a moist wound environment, assisting in proper wound bed preparation, and promoting wound healing[81]. They maintain a moist wound environment which implies that they can be easily removed without pain. They are also used as outer dressings after application of topical antibiotics, such as metronidazole, or hydrogels. Polyurethane foam is widely used in diabetic foot wounds and is capable of absorbing light to heavy amounts of exudate, thereby preventing maceration, facilitating removal of slough, and promoting the proliferative stage of wound healing[82].

Hydrogel dressings

These dressings consist of cross-linked insoluble starch or carboxymethylcellulose polymers and water (96%). The term hydrogel implies that the material is already swollen in water. Hydrogels donate fluid to dry necrotic and slough wounds and promote autolysis. They apparently debride by rehydrating the wound. These dressings are the best choice for the treatment of dry wounds with necrotic eschar, and the hydrogel reaches a 50% debridement level more quickly than wet-to-dry dressings and are more cost-effective[83-85]. The hydrogel hydrates, cools the wound and provides an analgesic effect.

Hydrocolloid dressing

These dressings are a combination of polymers such as gelatin, pectin and cellulose which form a waterproof adhesive dressing. Exudates produced by the wound are absorbed into the dressing and form a gel. Hydrocolloid dressings are capable of absorbing low to moderate levels of exudate and can be used to promote autolytic debridement of dry, sloughy, or necrotic wounds[86]. They maintain a moist wound healing environment and promote autolytic debridement of necrotic and sloughing tissues. They can be used as occlusive dressings and are very good at absorbing exudate. Hydrocolloid dressings should be avoided on plantar ulcers of the foot, as the periwound skin is susceptible to maceration. Additionally, hydrocolloids have been shown to retain growth factors under the dressing as well as promote granulation and epithelialization[87]. The low pH created by the hydrocolloid is effective for the treatment of wounds infected by *Pseudomonas* species[88].

Alginate dressings

Alginate dressings are highly absorbent and are available in two forms; calcium alginate and calcium sodium alginate. The use of alginate dressings as hemostatic agents was reported both *in vitro* and in clinical studies. The selection of an alginate dressing is usually to manage wound exudate, as it is claimed that they can absorb 15-20 times their own weight in wound fluid[89]. The alginate forms a gel when it comes into contact with the wound surface. It can be used in granulating, epithelializing, and cavity wounds. Cochrane reviews detail the role of alginate dressings in the treatment of diabetic foot ulcers[90,91].

Growth factors

Growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor, transforming growth factor (TGF)-β, TGF-α, epidermal growth factor (EGF), *etc*., are very effective in diabetic wound healing and have been reported to accelerate the formation of various components of healing. Growth factors stimulate different functions including angiogenesis, enzyme production, cell migration, and cellular proliferation[92]. Diabetic wounds are enriched in proteases and supports the premise that impaired growth factor availability may act as a rate limiting factor in diabetic wound healing[93]. PDGF regulates cell growth and division. It plays a significant role in blood vessel formation (angiogenesis). A recombinant human (rh)-PDGF dressing is an effective modality for facilitating wound healing in patients suffering from diabetes and can be used as an adjunct to the conventional mode of treatment for healing diabetic wounds[94]. It can be used in the granulating stage of the wound. EGF stimulates the proliferation of fibroblasts, keratinocytes, and vascular endothelial cells, which contributes to scar tissue formation. Local injections of rh-EGF offer a favorable risk-benefit balance in patients with advanced diabetic foot ulceration and was significantly enhanced by 75 μg EGF treatment in neuropathic *vs* ischemic ulceration[95].

Honey-impregnated dressings

Proposed to have antimicrobial and anti-inflammatory properties, these dressings can be used for acute or chronic wounds. The antimicrobial properties of honey have been demonstrated in the laboratory, however, *in vivo* evidence is scant, particularly in comparison to the literature on silver antimicrobial dressings[96,97].

Topical enzymes

Collagenase, fibrinolysin, or papain containing ointments help in the enzymatic debridement of sloughy tissues and thus promote granulation formation. Collagenase and papain/urea formulations have been demonstrated to have degrading effects on wound components, such as collagen, fibrin, and elastin both *in vitro* and clinically. Papain-urea and collagenase have proven efficacy in enzymatic wound debridement. Papain-urea (89.2%) is a better enzymatic debriding agent than collagenase (82.2%)[98].

Mechanical device

Vacuum-assisted closure generates a topical negative pressure over the wound bed. Pressure of 125 mmHg is the ideal pressure. Vacuum-assisted closure is extremely effective in removing exudate and reducing edema, while leaving the surface of the wound moist. It is contraindicated in avascular wounds or exposed tendons or bones. Some of the contraindications include untreated osteomyelitis,

non-enteric and unexplored fistula, presence of necrotic tissue, exposed organs or blood vessels, and malignancy in the wound[99]. Vacuum-assisted closure is effective in promoting wound closure in patients with treated osteomyelitis or soft tissue infections[100,101]. Hyperbaric oxygen therapy (HBOT) is another treatment which is used as an adjunct to standard wound care in the treatment of diabetic foot wounds. It has limited side effects, is relatively safe, and is widely used[102].

CONCLUSION

The successful management of diabetic foot wounds requires the multidisciplinary teamwork of specialists. The management of diabetic foot wounds needs timely detection of complications and frequent assessment of the wound. No wound should be treated as simple. It is important to take into account all the related causes, identify the problem, and treat it. There are various topical regimes available, but the choice depends only on the treating physicians, podiatrist, or clinical care nurse. While selecting wound care materials one should bear in mind the properties of the ideal wound care dressing which should maintain a moist wound healing environment, absorb exudates, control infection/odor and be effective in treating diabetic foot wounds. In addition to these wound care techniques, antibiotic therapy and offloading plays a very important role.

ACKNOWLEDGMENTS

We acknowledge Mrs. Spandana Birajdar of the Publication Department of Chellaram Diabetes Institute for providing writing assistance and language support.

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P- Reviewer: Romani A, Sriraman R, Surani S, Tamemoto H
S- Editor: Song XX **L- Editor**: Webster JR **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (3): Type 1 diabetes

Hepatitis C virus infection and type 1 and type 2 diabetes mellitus

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Received: November 29, 2013 Revised: April 10, 2014
Accepted: July 12, 2014
Published online: October 15, 2014

Abstract

Hepatitis C virus (HCV) infection and diabetes mellitus are two major public health problems that cause devastating health and financial burdens worldwide. Diabetes can be classified into two major types: type 1 diabetes mellitus (T1DM) and T2DM. T2DM is a common endocrine disorder that encompasses multifactorial mechanisms, and T1DM is an immunologically mediated disease. Many epidemiological studies have shown an association between T2DM and chronic hepatitis C (CHC) infection. The processes through which CHC is associated with T2DM seem to involve direct viral effects, insulin resistance, proinflammatory cytokines, chemokines, and other immune-mediated mechanisms. Few data have been reported on the association of CHC and T1DM and reports on the potential association between T1DM and acute HCV infection are even rarer. A small number of studies indicate that interferon-α therapy can stimulate pancreatic autoimmunity and in certain cases lead to the development of T1DM. Diabetes and CHC have important interactions. Diabetic CHC patients have an increased risk of developing cirrhosis and hepatocellular carcinoma compared with non-diabetic CHC subjects. However, clinical trials on HCV-positive patients have reported improvements in glucose metabolism after antiviral treatment. Further studies are needed to improve prevention policies and to foster adequate and cost-effective programmes for the surveillance and treatment of diabetic CHC patients.

Key words: Hepatitis C virus infection; Type 1 diabetes mellitus; Type 2 diabetes mellitus; Epidemiology; Pathogenesis; Prevention; Treatment

Core tip: Many studies have shown an association between type 2 diabetes mellitus (T2DM) and chronic hepatitis C (CHC) infection. The processes through which CHC is associated with T2DM seem to involve direct viral effects, insulin resistance, proinflammatory cytokines, and chemokines. Few data have been reported on the association of CHC and T1DM. A small number of studies indicate that interferon-α therapy can induce T1DM. Diabetic CHC patients have an increased risk of developing cirrhosis and hepatocellular carcinoma compared with non-diabetics. Clinical trials on hepatitis C virus-positive patients have reported improvements in glucose metabolism after antiviral treatment.

Original sources: Antonelli A, Ferrari SM, Giuggioli D, Di Domenicantonio A, Ruffilli I, Corrado A, Fabiani S, Marchi S, Ferri C, Ferrannini E, Fallahi P. Hepatitis C virus infection and type 1 and type 2 diabetes mellitus. *World J Diabetes* 2014; 5(5): 586-600 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/586.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.586

INTRODUCTION

Hepatitis C virus (HCV) infection and diabetes mellitus (DM) are two major public health problems that cause devastating health and financial burdens worldwide[1,2]. Diabetes can be classified into two major types: type 1 (T1DM) and T2DM[3,4]. T2DM is a common endocrine disorder that encompasses multifactorial mechanisms. These mechanisms include resistance to the action of insulin, increased hepatic glucose production, and a defect in insulin secretion, all of which contribute to the development of overt hyperglycaemia[5]. T1DM is an immunologically mediated disease. Prevention and treatment of T1DM are hampered by the fact that the key immunological mechanisms of the pathogenesis of the disease are still under debate[6,7]. However, a Th1 immune response is involved in β-cell destruction[8] and the importance of islet autoantibodies has been highlighted[9-11].

Chronic hepatitis C (CHC) infection has a global prevalence of 2%-3%. Approximately 170 million people are thought to be currently infected (approximately 3% of the world's population), and an additional 3-4 million are infected each year[12,13]. HCV is the main reason for liver transplantation in the developed world and the main cause of liver-related morbidity and mortality in a number of countries, including Italy. This virus is not only a frequent cause of chronic liver diseases, including hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), but it is also involved in the pathogenesis of various autoimmune and rheumatic disorders (*e.g.*, arthritis, vasculitis, sicca syndrome, porphyria cutanea tarda, lichen planus, nephropathies, and lung fibrosis) and in the development of B-cell lymphoproliferative diseases[14,15].

CHC is a multifaceted disorder that is associated with extrahepatic manifestations, including endocrinological disorders, thyroid disorders and diabetes[16,17].

In this paper, we review the increasing evidence linking HCV infection and DM in multiple fields (epidemiology, pathogenesis, clinical aspects, prevention, and treatment).

RELATIONSHIP BETWEEN CHC AND THE DEVELOPMENT OF T2DM

Origins of the hypothesis and epidemiological data in the general population

The liver plays an important role in carbohydrate metabolism, and liver diseases such as chronic hepatitis and cirrhosis are associated with a higher prevalence of disturbed glucose homeostasis, impaired glucose tolerance, and insulin resistance (IR)[18,19], which can eventually lead to DM[20-23]. Asymptomatic, moderate serum aminotransferase elevation has frequently been found in patients with DM, particularly in those with T2DM[24,25]. This phenomenon has often been related to fatty infiltration of the liver without further investigation[26,27]. In particular, steatosis has been related to IR and T2DM, beyond intracellular fat accumulation[28].

Liver fibrosis progression has also long been considered to be responsible for the development of IR and T2DM in patients with chronic liver diseases[29]. However, diabetes often occurs in the early stages of liver disease[30].

The aetiological factors that underlie the development of glucose homeostasis alterations were initially thought to be exclusively related to general long-term hepatocyte damage. However, later studies showed that patients with hepatitis B virus infection have a lower prevalence of T2DM compared with HCV-infected patients[31,32]. Thus, the question is as follows: "Does HCV infection itself have diabetogenic action?"

Since the discovery of HCV in 1989, attention has been paid to the association of CHC with the development of DM. Additionally from 1994[33] until now, several epidemiological studies on the seroprevalence of HCV have shown higher prevalences in diabetic patients than in controls (Figure 1). Moreover, analyses have shown a higher prevalence of DM in patients who are seropositive for HCV than in controls without HCV infection.

To analyse the epidemiological data, we searched for published studies in the PubMed database, covering the period from 1994 to December 2012. The literature search was performed using combinations of the terms "diabetes", "diabetes mellitus", "type 2 diabetes mellitus", "T2DM", "type 2 DM", "non-insulin dependent diabetes", or "NIDDM"; "hepatitis", "hepatitis C", "hepatitis C virus", "HCV", "HVC", or "chronic hepatitis"; and "risk", "risk factor", "case-control", "cohort", "clinical trial", "cross sectional", "epidemiology", "observational", "meta-analysis", "systematic review", or "review". For epidemiological studies, we only searched human studies and publications in English and Italian, the languages understood by the authors.

The data represent a very heterogeneous population regarding gender, age, and ethnic group. Globally, approximately seventy studies are in agreement with an association[18,26,30-96], although not all of them have shown significant data. However, some of the non-significant data may be attributed to small sample sizes and other methodological factors (Figure 1).

Certain negative data that are not in agreement with an association between HCV infection and T2DM have also been reported[97-104]. However, the number of published epidemiological studies that are in agreement with the association between HCV infection and T2DM is higher than the number of studies in disagreement with this hypothesis.

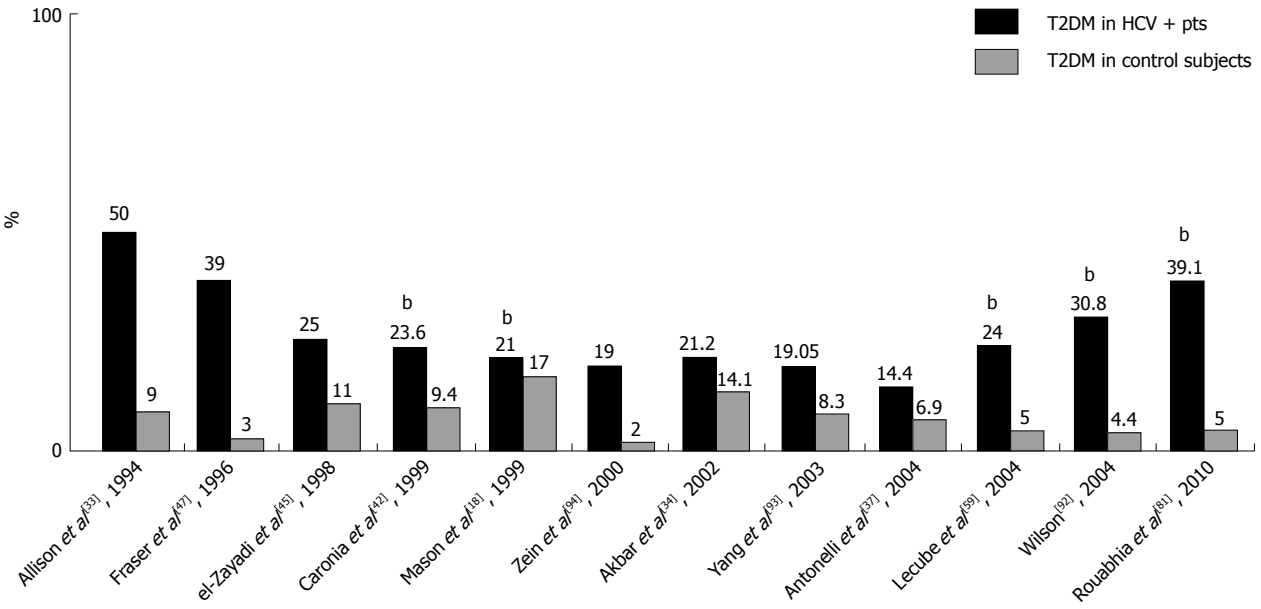

Figure 1 Patients seropositive for hepatitis C virus show a higher prevalence of diabetes mellitus than healthy controls. Twelve representative epidemiological studies demonstrated a relationship between HCV infection and the development of type 2 diabetes mellitus (T2DM). Analyses have shown a higher prevalence of diabetes mellitus in patients who are seropositive for HCV than in controls. $^{b}P < 0.001$, T2DM in HCV+ pts *vs* T2DM in control subjects. HCV+: Hepatitis C virus-infected; pts: Patients.

HCV INFECTION AND T2DM ASSOCIATION: PATHOGENESIS

Direct effects of HCV and IR

HCV is hepatotropic and noncytopathic; nevertheless, its genome has been identified in a number of tissues beyond the liver, including pancreatic acinar cells and epithelial cells of the pancreatic duct[105,106]. Although post-mortem studies have revealed that HCV replicates in the pancreas[107] and animal models have suggested a direct effect of HCV infection on IR in the liver[108], the evidence is scanty.

Of interest are the roles of structural and non-structural HCV proteins. HCV has an RNA genome of 9.6 kb that encodes approximately 3010 amino acids and is translated into structural (core, E1, and E2) and non-structural (NS3-NS5B) proteins. These proteins play a role in the development of IR and oxidative stress *via* reactive oxygen species at the cellular level[109-113]. The HCV core protein, alone or in combination with other viral proteins, increases phosphorylation of insulin receptor substrate-1 (IRS-1), which is the basis of IR[114-116]. Phosphorylated IRS-1 activates phosphatidylinositol 3-kinase (PI3K)[117,118], and the activation of PI3K and one of its downstream targets, Akt, is essential for most of the metabolic effects of insulin[119-126]. Therefore, defects at the level of the association of PI3K with IRS-1 and a lack of PI3K activation may contribute to IR and the increased prevalence of diabetes in HCV-infected patients. Indeed, this mechanism ultimately promotes glucose transporter-4 translocation to the plasma membrane to enhance glucose uptake[127,128]. Within the IR mechanism impairment of the activation of Akt/PKB is the key step that can inhibit glucose uptake[30,129,130].

The detailed molecular events leading to IR in HCV-infected patients are, however, unclear. Recent evidence supports the existence of a significant extrahepatic component of HCV-induced IR. Thus, the molecular pathogenesis of the glucose metabolism disturbances observed in hepatitis C is much more complex than expected[131].

Recently, Eslam *et al*[132] showed that polymorphisms in the IFNL3 (IL28B) region are associated with spontaneous and treatment-induced recovery from HCV infection. Furthermore, circumstantial evidence suggests a link between single-nucleotide polymorphisms in IFNL3 and lipid metabolism, steatosis, and IR in CHC. The emerging picture suggests that the responder genotypes of IFNL3 polymorphisms are associated with higher serum lipid levels and less frequent steatosis and IR[132].

HCV-induced immune responses; cytokines, chemokines-mediated effects

Viral innate immune evasion strategies and human genetic determinants underlie the transition of acute HCV infection into viral persistence and chronic infection. Host genetic factors can influence both the outcome of the infection and the response to antiviral therapy. Recent insights into how HCV regulates immune signalling within the liver reveal a complex interaction of the patient's genetic background with viral and host factors related to the innate immune triggering and control that dictate the outcome of HCV infection and immunity[133].

Beyond the direct effects of HCV on IRS-1/PI3K, the HCV core protein may induce IR indirectly *via* stimulation of the secretion of proinflammatory cytokines[115]. In patients with CHC, most likely due to HCV-induced inflammation, there is hypersecretion of insulin-resistant proinflammatory cytokines such as interleukin (IL)-6 and

tumour necrosis factor (TNF)-α[134-138]. Proinflammatory cytokines also upregulate suppressors of cytokine signalling proteins as part of a negative feedback loop to attenuate cytokine signalling[139,140]. This phenomenon may contribute to increased gluconeogenesis due to a lack of Akt-mediated inhibition of phosphoenolpyruvate carboxykinase gene expression. In this context, it is interesting to note that leptin can modulate the action of insulin in liver cells by antagonising insulin-stimulated IRS-1 tyrosine phosphorylation, increasing phosphoenolpyruvate carboxykinase gene expression, and decreasing glucokinase expression, which results in increased gluconeogenesis[141]. Together with the increase in gluconeogenesis, the enhanced production and accumulation of lipids mediated by inhibition of the AMP-activated protein kinase occur after HCV infection[142]. Additionally TNF-α plays a role in lipid metabolism. Indeed, the lipolysis-stimulating effect of TNF-α leads to increased serum levels of free fatty acids, which reduces insulin sensitivity[143,144].

Cytokines are intercellular mediators involved in viral control and in the liver damage induced by infection with HCV. The complex cytokine network that operates during the initial infection allows the coordinated, effective development of both the innate and the adaptive immune responses. However, HCV interferes with cytokines at various levels and escapes the immune response by inducing a Th2/T cytotoxic 2 cytokine profile. The inability to control infection leads to the recruitment of inflammatory infiltrates into the liver parenchyma by interferon (IFN)-γ-inducible CXC chemokine ligand (CXCL)9, CXCL10, and CXCL11, which result in sustained liver damage and eventually liver cirrhosis. The most important systemic HCV-related extrahepatic diseases (mixed cryoglobulinemia, lymphoproliferative disorders, thyroid autoimmune disorders, and T2DM) are associated with complex dysregulation of the cytokine/chemokine network, involving proinflammatory and Th1 chemokines[145,146].

HCV-INFECTED PATIENTS WITH T1DM

Few data on this association have been reported, and published studies have shown only small proportions of CHC patients positive for one or more markers of pancreatic autoimmunity[18,147-150].

Even rarer are reports on the potential association between autoimmune diabetes and acute HCV infection. Only two cases have been described in the literature[151,152]. Several mechanisms have been postulated to initiate the process. Even if HCV can infect extrahepatic tissue in patients with hepatitis C[16,107,153], no direct involvement of HCV in the onset of T1DM has been clarified yet. Nevertheless, the direct destruction of β-cells by viral infection could be a good explanation. Beyond the undemonstrated direct mechanisms, HCV infection surely initiates an immune reaction against β-cells or causes an acceleration of diabetes onset when an immune reaction against β-cells is already present. Some authors have also suggested the involvement of a process of molecular mimicry as a trigger of HCV-related autoimmunity[154,155]. Indeed, glutamic acid decarboxylase (GAD) 65 shares amino acid sequence similarities with antigenic regions of the HCV polyprotein[156]. Of interest, HCV/self-homologous autoantigenic regions are also mimicked by other microbial agents. Such mimics may give rise to β-cell autoimmunity through a multiple-hit mechanism of molecular mimicry[154,155,157]. Cross-reactive immunity does not exclude the possible involvement of additional factors, such as proinflammatory cytokines, which may act in concert, leading to the development and/or maintenance of pancreatic autoimmunity during acute HCV infection[156]. Another possibility is the induction of antibody reactivity against GAD and the development of full-blown diabetes, mediated by IL-18 and other proinflammatory cytokines. In particular, IL-18 is presumed to play a pathogenetic role in T1DM, specifically because this cytokine appears to be involved in acceleration of the development of overt disease[152,158-160]. IL-18 can induce both Th1 and Th2 responses, depending on the surrounding cytokines[161], and this cytokine plays a pathogenic role in several diseases[161], including acute hepatic injury[162]. Other proinflammatory cytokines, such as TNF-α and IL-1β, which are elevated in patients with acute hepatitis[163], can also induce autoimmune diabetes[164-167].

OTHER IMMUNE ASPECTS OF HCV ASSOCIATED WITH T1DM OR T2DM

Immune aspects have been reported in both T1DM and T2DM, and based on the immunology, it is clear that the lines separating T1DM from latent autoimmune diabetes in adults (LADA) and T2DM are not well delineated[10,11,16,37,145,168-170].

The type of diabetes manifested by patients with CHC is not classical T2DM, and the labelling of HCV patients as having T2DM is purely conventional and possibly inaccurate. The lines separating T1DM from LADA and T2DM are fading away as new pathogenetic information is obtained[170].

Three studies have reported[37,38,171] that HCV patients with T2DM are leaner than T2DM controls and show significantly lower low-density lipoprotein-cholesterol levels and systolic and diastolic blood pressures. Furthermore, patients with HCV-associated mixed cryoglobulinaemia (MC + HCV) and T2DM had non-organ-specific autoantibodies more frequently (34% *vs* 18%, respectively) than did non-diabetic MC + HCV patients[37]. An immune-mediated mechanism for MC + HCV-associated diabetes has been postulated[37], and a similar pathogenesis might be involved in diabetes in HCV patients. This hypothesis is strengthened by the finding that autoimmune phenomena are more common in T2DM patients than previously thought[10]. However, as the prevalence of classic β-cell autoimmune markers is not increased in HCV patients[70], other immune phenomena might be involved[168]. Chemokines could be important in this context. In fact, in children with newly

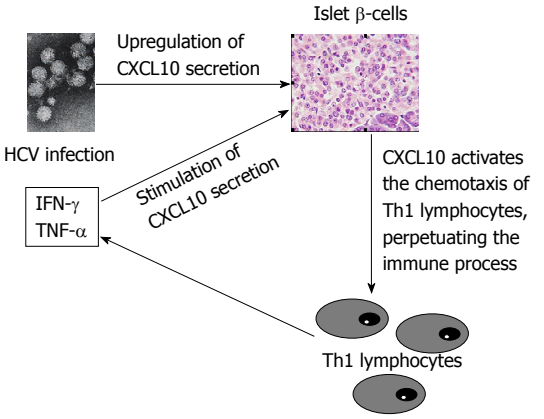

Figure 2 Potential regulation of the endocrine manifestations of hepatitis C virus infection in islet β-cells. Hepatitis C virus (HCV) infection may act by upregulating CXC chemokine ligand (CXCL) 10 gene expression and the subsequent secretion of this chemokine by islet β-cells. These events lead to the recruitment of Th1 lymphocytes that secrete interferon (IFN)-γ and tumour necrosis factor (TNF)-α, which induce chemokine secretion by islet β-cells, thus perpetuating the immune cascade. This cascade may lead to the appearance of autoimmune thyroid disorders in genetically predisposed subjects.

diagnosed T1DM, raised serum CXCL10 and normal chemokine (C-C motif) ligand 2 concentrations signal a predominantly Th1-driven autoimmune process, which shifts toward Th2 immunity 2 years after diagnosis[172].

Based on the abovementioned concepts, HCV infection of β-cells[106] may act by upregulating *CXCL10* gene expression and secretion (as previously shown in human hepatocytes[173]) and recruiting Th1 lymphocytes that secrete IFN-γ and TNF-α, which induce CXCL10 secretion by β-cells and thus perpetuate the immune cascade. This cascade may lead to the appearance of β-cell dysfunction in genetically predisposed subjects (Figure 2). Recently, certain studies have confirmed this hypothesis, demonstrating higher serum levels of CXCL10 in HCV patients with T2DM than in those without[16,169].

T1DM AND T2DM IN HCV-INFECTED PATIENTS TREATED WITH IFN-α

An important research area concerns the relationship between diabetes and IFN-α therapy in HCV-infected patients. In particular, studies have shown a high prevalence of markers of pancreatic autoimmunity in HCV-positive patients after or during IFN-α therapy, most likely due to the immunostimulatory effects of this cytokine. Indeed, IFN-α has antiviral, antiproliferative, and immunomodulatory activities[174]. Thus, in predisposed individuals, IFN-α can either induce a diabetogenic process or accelerate a diabetogenic process that is already underway[18,175,176]. For this reason, islet cell autoantibodies and GADAb should be investigated before and during IFN treatment to identify subjects who are at high risk of developing T1DM[177-180]. A small number of patients can develop *de novo* pancreatic autoimmunity and fall into a group of patients at risk of developing DM. In general, patients who are initially positive for organ-specific autoantibodies (in particular, thyroid- and pancreas-specific autoantibodies) and those who seroconvert seem to be at high risk of developing clinical autoimmune disease after treatment with IFN-α[181]. Timely suspension of IFN-α therapy is rarely accompanied by regression of clinical DM. No correlation has been documented between the response to antiviral therapy and the development of DM.

IFN-α increases HLA class I antigen expression and natural killer cell and T cell activities, and this cytokine may be an important cofactor in the development of a Th1 immune reaction. This reaction can contribute to the development of autoimmune disease by the activation of CD4+ lymphocytes that secrete IL-2, IFN-γ and TNF-β. These cytokines help in the generation of CD8+ cytotoxic T cells[182]. In addition to its immunomodulatory properties, IFN-α can also increase IR and induce hyperglycaemia[183-188]. Fabris *et al*[189] documented the first case of T1DM development during IFN-α therapy. Other studies suggest that IFN-α therapy can stimulate pancreatic autoimmunity and, in certain cases, lead to the development of T1DM[150,175,177,180,181,190-223].

The relationship with T1DM does not account for all of the effects of IFN-α therapy on diabetes. Indeed, from a completely different perspective, antiviral therapy with IFN should also be considered in HCV-positive patients because of its potential role in limiting the progression of this metabolic disturbance (see later discussion).

OUTCOME IN DIABETIC HCV-POSITIVE PATIENTS

CHC is an insidiously progressive form of liver disease that leads to cirrhosis[224-226] and HCC[227-231]. Diabetic HCV-positive patients have increased risk compared with non-diabetic subjects, and DM itself seems to have a selective impact on HCC development[232-251].

The main characteristic of diabetic patients is IR, which plays a crucial role in fibrosis progression and has a negative impact on treatment responses to antiviral therapy in patients with CHC[52,252,253]. Reduced insulin sensitivity is at the basis of compensatory hyperinsulinemia and elevated levels of insulin-like growth factor 1 (IGF-1), which stimulates cell proliferation and inhibits apoptosis. Additionally, this phenomenon has strong mitogenic effects on a wide variety of cancer cell lines[254-256]. At the same time, insulin activates the IGF-1 receptor, which has a growth-promoting effect that includes modulating cell cycle progression. Excess insulin may also indirectly affect the development of cancer by downregulating the level of IGF-binding protein 1, which increases the level and bioavailability of total circulating IGF-1. Additional factors, such as obesity and physical inactivity, also cause hyperinsulinemia and are thus also ultimately associated with accelerated cancer progression[255-258].

Genotype differences in terms of liver disturbance progression have been described as well. Genotype 3a is more strongly correlated with steatosis than other

genotypes[259,260], and the HCV genotype 3 may have a cytopathic effect[261]. Steatosis in genotype 1 infection is instead thought to be an expression of metabolic syndrome caused by the activation of proinflammatory mechanisms as well as underlying obesity and IR[262]. The degree of steatosis in this genotype is independent of the HCV viral load, and antiviral therapy does not improve steatosis in these patients. Similar data have been obtained for genotype 4 infection, whereas few data are available for genotype 2[263].

The presence of HCV infection in patients with DM may also increase the proportion of DM-related chronic nephrologic complications[86,264].

PREVENTION AND TREATMENT

CHC is a complex disease with systemic effects that require a multidisciplinary treatment approach[265].

The potential relationship between HCV infection and the development of DM increases the need for the implementation of prevention measures. Prevention must be directed toward lifestyle changes that can reduce the risk of HCV infection and/or diabetes development[266]; regular diabetes screening for anti-HCV-positive people; and the analysis of other risk factors that can accelerate the progression of both CHC and DM, such as obesity, dyslipidaemia, and alcohol consumption. In these high-risk patients, comprehensive treatment, including lifestyle modifications, must be recommended. Animal models also provide clues regarding the prevention and clinical management of diabetes in the setting of HCV infection[108]. Indeed, identifying patients who are at risk of developing diabetes, and have CHC, reduces liver disturbance progression[267,268], the incidence of HCC and transplant-related morbidity and mortality. Additionally, this identification improves the response to antiviral therapy[269-271], even reducing the side effects of the treatment[270] by encouraging the pretreatment of IR and DM[265].

Moreover, clinical trials on HCV-positive patients have reported improvement in glucose metabolism after antiviral treatment[187]. As discussed earlier, many factors may surely affect the antiviral response that modulates the IFN signalling pathway. Among these factors, the HCV genotype, genetic host factors, and comorbidities have been taken into account. In particular, recent studies have reported obesity[272] and hypercholesterolaemia[273] as potential factors that interfere with a sustained viral response. These observations suggest additional therapeutic options for HCV infection, including dietary changes, anti-diabetic drugs, and statins. Concerning anti-diabetic drugs, it is not currently clear whether the best approach is to use a peroxisome proliferator-activated receptor agonist or a biguanide, such as metformin[274-276]. Concerning statins, these drugs are capable of inhibiting HCV replication *in vitro*[277-279] but not *in vivo*[280].

Further studies are needed to improve prevention policies and to foster adequate and cost-effective programmes for the surveillance and treatment of diabetic CHC patients. The final goal must be to cure two diseases, diabetes and CHC, with one multifaceted treatment.

CONCLUSION

Many epidemiological studies have shown an association between T2DM and CHC. The processes through which HCV is associated with DM seem to involve direct viral effects, IR, proinflammatory cytokines, chemokines, suppressors of cytokine signalling, and other immune-mediated mechanisms. Other factors, such as metabolic syndrome and a family history of diabetes, also seem to be important risk factors for the development of diabetes. Few data on the association of CHC and T1DM have been reported, and reports on the potential association between T1DM and acute HCV infection are even rarer. A small number of studies have indicated that IFN-α therapy can stimulate pancreatic autoimmunity and, in certain cases, lead to the development of T1DM. Diabetes and CHC have important interactions. Diabetic CHC patients have an increased risk of developing cirrhosis and HCC compared with non-diabetic CHC subjects. Additionally, clinical trials on HCV-positive patients have reported improvement in glucose metabolism after antiviral treatment. Further studies are needed to improve prevention policies and to foster adequate and cost-effective programmes for the surveillance and treatment of diabetic CHC patients.

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P- Reviewer: Dashora U **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Targeting inflammation in diabetes: Newer therapeutic options

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Received: December 27, 2013 Revised: April 24, 2014
Accepted: May 29, 2014
Published online: October 15, 2014

Abstract

Inflammation has been recognised to both decrease beta cell insulin secretion and increase insulin resistance. Circulating cytokines can affect beta cell function directly leading to secretory dysfunction and increased apoptosis. These cytokines can also indirectly affect beta cell function by increasing adipocyte inflammation.The resulting glucotoxicity and lipotoxicity further enhance the inflammatory process resulting in a vicious cycle. Weight reduction and drugs such as metformin have been shown to decrease the levels of C-Reactive Protein by 31% and 13%, respectively. Pioglitazone, insulin and statins have anti-inflammatory effects. Interleukin 1 and tumor necrosis factor-α antagonists are in trials and NSAIDs such as salsalate have shown an improvement in insulin sensitivity. Inhibition of 12-lipooxygenase, histone de-acetylases, and activation of sirtuin-1 are upcoming molecular targets to reduce inflammation. These therapies have also been shown to decrease the conversion of pre-diabetes state to diabetes. Drugs like glicazide, troglitazone, N-acetylcysteine and selective COX-2 inhibitors have shown benefit in diabetic neuropathy by decreasing inflammatory markers. Retinopathy drugs are used to target vascular endothelial growth factor, angiopoietin-2, various proteinases and chemokines. Drugs targeting the proteinases and various chemokines are pentoxifylline, inhibitors of nuclear factor-kappa B and mammalian target of rapamycin and are in clinical trials for diabetic nephropathy. Commonly used drugs such as insulin, metformin, peroxisome proliferator-activated receptors, glucagon like peptide-1 agonists and dipeptidyl peptidase-4 inhibitors also decrease inflammation. Anti-inflammatory therapies represent a potential approach for the therapy of diabetes and its complications.

Key words: Inflammation; Insulin resistance; Diabetes; Neuropathy; Retinopathy; Nephropathy

Core tip: The burden of diabetes and its complications is increasing worldwide. To control this pandemic, drugs targeting different areas of the pathogenesis of diabetes and its complications are needed. Inflammation plays a key role in the natural history of diabetes during the progression from pre-diabetes to diabetes, including decreased beta cell secretory capacity and insulin resistance. Insulin resistance is an important part of the metabolic syndrome and plays a role in the pathogenesis of various macrovascular complications. Drugs targeting inflammatory pathways represent a fresh approach in the treatment of diabetes and its complications.

Original sources: Agrawal NK, Kant S. Targeting inflammation in diabetes: Newer therapeutic options. *World J Diabetes* 2014; 5(5): 697-710 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/697.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.697

INTRODUCTION

The incidence of both diabetes and obesity is increasing worldwide and approaching epidemic proportions. Inflammation has been recognised as a common mechanism in the pathophysiology of both these conditions. Inflammation increases insulin resistance and islet cell inflammation, which leads to defects in beta cell secretion both of which lead to diabetes. Inflammation may also be the underlying mechanism in the increased risk of cardiovascular disease in subjects with diabetes and/or obesity. Hence, targeting inflammation may be a new therapy in the already expanding options for the management of diabetes mellitus and its complications. There is concern over many drugs used for diabetes which increase cardiovascular morbidity and/or mortality. Targeting inflammation in diabetes will theoretically lead to better glycemic control, and decrease both micro- and macrovascular complications including cardiovascular complications. Most therapies for type 2 diabetes mellitus (T2DM) target insulin resistance and drugs targeting inflammation may be a paradigm shift, wherein earlier recognition of the inflammatory status of the predisposed individual with type 2 diabetes, or at risk for the development of type 2 diabetes, would be evaluated and appropriate therapy initiated.The aim of this review is to elaborate on the drugs targeting inflammation in diabetes and its complications. Both previous studies and upcoming targets including their molecular mechanisms will be discussed in the review.

Inflammation in diabetes

A number of studies have demonstrated that markers of inflammation correlate with incident diabetes. Total leucocyte count which is a surrogate marker of inflammation, and more specifically the neutrophil count in the higher quartiles of the normal range, correlates with worsening of insulin sensitivity, and incident diabetes[1] and cardiovascular disease[2]. This suggests that a simple surrogate marker such as total leucocyte count may be a marker of insulin resistance.

Insulin resistance has been defined as a state of inflammation involving both innate and adaptive immunity[3]. Islet cell inflammation as a result of an autoimmune phenomenon has already been recognised in T1DM and has been increasingly implicated in the pathogenesis of T2DM. In fact, obesity has also been seen to modify the development of T1DM. Small human studies have demonstrated that anti-inflammatory therapy has improved glycemia and beta cell function in T2DM[4,5]. Thus, inflammation is recognised as one of the important pathways in the pathogenesis of T2DM and its complications.

The major cell involved in inflammation and insulin resistance in T2DM is the adipocyte. Insulin regulates glucose uptake and triglyceride storage by adipocytes. The adipocytokines in turn also affect insulin secretion and insulin resistance[6,7]. The various adipocytokines, especially leptin, adiponectin, omentin, resistin, and visfatin may contribute to beta cell dysfunction by increasing insulin resistance. Adipose tissue also secretes dipeptidyl peptidase-4 (DPP-4) which enhances the degradation of glucagon like peptide-1 (GLP-1) and has an insulinotropic effect on beta cells[8].

Circulating cytokines can affect beta cell function directly and indirectly by increasing adipocyte inflammation. Cytokines including tumour necrosis factor-alpha (TNF-α), interleukin beta (IL-1β), and interferon-gamma (IFN-γ) disrupt the regulation of intracellular calcium in the beta cells and hence insulin release. In addition, TNF-α increases the expression of islet amyloid polypeptide (IAPP, amylin) in beta cells leading to their accelerated death[9]. IAPP expression and deposition induces and increases beta cell inflammation[10,11]. Glucotoxicity and especially lipotoxicity increase the local level of free fatty acids (FFA) in the islets, and long chain fatty acids, particularly palmitic acid, cause oxidative stress and jun N-terminal kinase (JNK) activation[12]. This further leads to increased IL-1β, TNF-α, chemokine (C-C motif) ligand 2 (CCL2), IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1), and IL-8 production, and activated nuclear factor-kappa B (NF-κB) in human islets leading to islet cell dysfunction[13]. Overall, this leads to a vicious cycle of inflammation-induced beta cell dysfunction which in turn again increases inflammation.

Oxidative stress is another pathway that leads to inflammation through activation of JNK, NF-κB, and p38 mitogen-activated protein kinase (p38MAPK)[14]. Palmitic acid causes endoplasmic reticulum (ER) stress, oxidative stress, ceramide production, and JNK activation, all of which provoke inflammatory responses. Pancreatic islets have low antioxidant defence and are hence vulnerable to oxidative stress. There is differential regulation of oxidative stress genes in T2DM donors compared with control subjects, implicating oxidative stress in islet dysfunction[15]. Divalent metal transporter 1 is another factor that increases IL-1β-induced insulin resistance[16]. These findings suggest that oxidative stress is an important factor in the pathogenesis of T2DM.

Endoplasmic reticulum stress also leads to increased cytokine expression and NF-κB activation causing dysfunction of beta cells[17]. Infact, cyclopiazonic acid-induced ER stress has been shown to cause beta cell dysfunction through increased levels of cytokines and NF-κB expression[18]. The levels of thioredoxin-interacting protein (TXNIP) increase rapidly in islets during ER stress provoked by thapsigargin (depletes calcium stores in the ER). Up-regulation of TXNIP results in IL-1β and IL-6 production through initiation of the inflammasome[19,20]. TXNIP also leads to induction of oxidative stress through its interaction with thioredoxin, which is a critical redox protein in cells. TXNIP expression is regulated by glucose in human islets and plays a role in glucose-induced β cell death.Therefore, TXNIP may well be a key transducer of glucotoxicity, oxidative stress, and ER stress, feeding into various inflammatory pathways in islets.

The gut may also be involved in the development of

diabetes mellitus. Increased lipopolysaccharide absorption from the gut causes activation of toll like receptor 4 and NF-κB leading to decreased insulin gene expression and insulin secretion in rat and human islets[21]. There is data to suggest that colonization of the gut by specific bacterial species alters the development of autoimmunity in NOD mice and can modify the cytokine and chemokine profile leading to islet cell inflammation[22].

With all this in mind, the search for anti-inflammatory therapies for diabetes was started. Lifestyle modification and drugs already in use for the management of diabetes also have additional anti-inflammatory effects. In the Diabetes Prevention Program (DPP), weight reduction decreased the levels of C-Reactive Protein (CRP) by 31%, whereas metformin decreased CRP by only 13%[23]. Similar results have been observed with surgical weight loss procedures[24]. This implies that lifestyle interventions, even without drug therapy, can decrease insulin resistance; and decrease the progression of pre-diabetes states to T2DM and can decrease the progression of diabetes mellitus (DM) and its complications by decreasing inflammation. Drugs like thiazolidinedione for the same degree of glucose reduction have been shown to reduce markers of inflammation to a greater extent compared to other therapies[25]. This may be the result of peroxisome proliferator-activated receptor-γ (PPAR-γ) transrepression of inflammatory-response genes[26]. This demonstrates that a reduction in inflammation adds to the beneficial effects of these drugs, which are independent of the effect on glucose levels and thus is a direct effect.

Insulin therapy by itself over the short-term has been associated with a decrease in inflammation. This effect is mediated by the decreased activity of NF-κB which is the master transcriptional regulator of the inflammatory response[27]. However, this effect of insulin is temporary and/or requires higher doses of intravenous insulin[28]. This may be one of the additional advantages of adding insulin early in the course of T2DM and may delay the progression of DM and its complications.

One class of drugs used widely in diabetes mellitus that also have anti-inflammatory effects are statins. Statins inhibit hydroxymethylglutaryl-CoA reductase, and hence, cause a reduction in cholesterol levels. In addition, statins have also been shown to reduce the levels of CRP by 25%-30%[29]. This is a class effect of all statins and is not dose-dependent. The decrease in CRP levels does not correlate with the decrease in lipid levels, which implies that this effect is a direct effect of statins. CRP is an independent predictor of cardiovascular events. The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin trial assessed the effect of rosuvastatin on the rates of primary cardiovascular events in subjects with high CRP concentrations, but without hyperlipidemia (CRP > 2 mg/L; low density lipoprotein (LDL) < 130 mg/dL)[30]. The CRP concentration was reduced by 37%, however, the LDL concentration was reduced by 50%, therefore, it is uncertain whether the effects of statins are truly mediated *via* the anti-inflammatory process or are the result of its lipid-lowering effect. In addition, incident T2DM increased in the statin-treated patients, an effect seen with other agents in the statin class[31]. This finding demonstrated a divide in the association between inflammation, diabetes, and cardiovascular disease, which may be explained by the potent effects of statins on lipids. Apart from CRP, statins do not have any effect on any other markers of inflammation such as fibrinogen.

NEWER THERAPEUTIC TARGETS

The following drugs are in trials for targeting inflammation and are not yet available as prescription drugs for diabetes.

Etanercept

Etanercept (934 amino acids, 150 kilo Dalton) is a dimeric fusion protein with an extracellular ligand binding domain of the Human Tumor Necrosis Factor Receptor (TNFR) linked to the Fc component of human IgG1. It is produced by a recombinant DNA technique in Chinese Hamster Ovary cells.

Blockade of TNF-α receptor has been shown to decrease insulin resistance in obese rats[32]. A trial of etanercept failed to improve insulin sensitivity in subjects with the metabolic syndrome despite lowering CRP[33]. This may have been due to the fact that the concentration of TNF-α intracellularly is almost twice that in the extracellular space, and it is the intracellular TNF-α that is responsible for insulin resistance *via* paracrine effects which were not blocked by etanercept.

Anakinra

Anakinra (153 amino acids, 17.3 kilo Dalton) is a non glycosylated form of the Human IL-1 Receptor antagonist (IL-1Ra) from which it differs only by the addition of a single methionine residue at the amino terminus. It is produced by a recombinant DNA technique in *E. coli*.

IL-1 contributes to impaired insulin secretion, decreased cell proliferation, and apoptosis of pancreatic β cells. The IL-1Ra is endogenously produced, and its concentrations are reduced in the pancreatic islets of patients with T2DM. Anakinra was studied in T2DM and showed promise in increasing beta cell secretory function, and reducing glycemia and markers of systemic inflammation[34]. Definitive conclusions on the possible clinical utility of IL-1Ra in the prevention of diabetes are awaited from the large ongoing Canakinumab Anti-inflammatory Thrombosis Outcomes Study phase Ⅲ clinical trial[35]. The study is being conducted in more than 40 countries around the world and is specifically testing whether blocking the pro-inflammatory cytokine IL-1β with canakinumab, as compared to placebo, can reduce rates of recurrent myocardial infarction, stroke, and cardiovascular death among patients with a history of myocardial infarction who remain at high risk due to a persis-

tent elevation of the inflammatory biomarker hsCRP (≥ 2 mg/L) despite best medical care.

Salsalates

Salsalates belong to the class of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) which exert their anti-inflammatory effect through inhibition of prostaglandin G/H synthase, or cyclooxygenase.These enzymes catalyse the transformation of arachidonic acid to prostaglandins and thromboxanes. NSAIDs also inhibit the expression of cell adhesion molecules, which play a role in targeting circulating cells to inflammatory sites and directly inhibit activation and function of neutrophils.

Trials with high dose salsalates in rodents[36] and in subjects with diabetes[37] have shown that salsalate by inhibiting the inhibitor of nuclear factor kappa-B kinase subunit beta decreases glucose intolerance and increases insulin sensitivity. In an open label study, salsalate, a prodrug form of salicylate, reduced fasting and post-challenge glucose levels and increased glucose utilization in euglycemic, hyperinsulinemic clamp studies[37]. Circulating FFAs were reduced and adiponectin levels were increased. In another study, salsalate, when compared with placebo, reduced fasting glucose by 13% ($P < 0.002$), glycemic response after an oral glucose challenge by 20% ($P = 0.004$), and glycated albumin by 17% ($P < 0.0003$). Although insulin levels were unchanged, fasting and oral glucose tolerance test and C-peptide levels decreased in the salsalate-treated subjects compared with placebo ($P < 0.03$), consistent with improved insulin sensitivity and a known effect of salicylates to inhibit insulin clearance. Adiponectin increased by 57% after salsalate treatment compared with placebo ($P < 0.003$). Additionally,within the group of salsalate-treated subjects, circulating levels of CRP were reduced by 34% ($P < 0.05$)[38]. These findings prove that salsalate reduces glycemia and may improve inflammatory cardiovascular risk indices in overweight individuals. These data support the hypothesis that sub-acute to chronic inflammation contributes to the pathogenesis of obesity-related dysglycemia and that targeting inflammation may provide a therapeutic option for diabetes prevention. However, the effects of salsalate on inflammation are controversial as shown by another study in which salsalate did not change flow mediated dilatation in peripheral conduit arteries in patients with T2DM despite lowering HbA1c. This finding suggests that salsalate does not have an effect on vascular inflammation[39].

Vitamin D

Calcitriol exerts regulatory effects on molecular pathways involved in inflammation, such as inhibition of PG synthesis and actions, inhibition of stress-activated kinase signaling and the resultant production of inflammatory cytokines,such as inhibition of NF-κB signaling and the production of pro-angiogenic factors. Clinical trials investigating the effects of vitamin D supplementation on serum levels of inflammatory markers have provided inconsistent results, with no evidence of effects in most trials, or effects on selected markers in a few other trials[40]. Similarly, available trials have shown no convincing benefits of vitamin D supplementation on plasma glucose levels and insulin resistance[41,42]. This systematic review and meta-analysis showed that vitamin D supplementation resulted in a small improvement in fasting glucose and insulin resistance in subjects with diabetes or impaired glucose tolerance, but no effect on glycated haemoglobin among those with diabetes. Hence, the role of vitamin D supplementation requires further well planned trials.

Chloroquine

Chloroquine is a weak base and carries a positive charge at acidic pH. It is this property of the drug that makes it selectively accumulate in lysosomes and generate a concentration gradient of a high order. This lysosomatotrophic action is responsible for the hepatic retention of insulin. Another action of the drug is decreased degradation of insulin in the muscle tissue.

A retrospective study suggested that the use of chloroquine to treat rheumatoid arthritis is associated with a lower incidence of T2DM[43]. However, this study included a specific group of patients who required the drug for another indication. Prospective studies of chloroquine are ongoing and the results are awaited.

Diacerin

Diacerin is a semi-synthetic anthraquinone derivative which directly inhibits IL-1 synthesis and release *in vitro* and downregulates IL-1 induced activities. It has been shown to possess a disease modifying effect in osteoarthritis.

In a randomized double-blind, placebo-controlled trial, 2-mo treatment of drug-naive T2DM patients with diacerin increased insulin secretion without changes in insulin sensitivity[44]. This implies a direct effect of the drug on beta cell function.

Other emerging therapies

Inhibition of 12-Lipo oxygenase: Twelve-Lipo oxygenase (12-LO) produces pro-inflammatory arachidonic acid products and is upregulated in islets of both T1DM and T2DM patients[45] leading to insulin resistance and islet cell dysfunction. Hyperglycemia and inflammatory cytokines increase the expression of 12-LO[45,46]. The activation of 12-LO has also been implicated in causing adipose tissue inflammation and insulin resistance. In NOD mice (T1DM model), Zucker diabetic fatty rats (T2DM model), and diet-induced obese mice (T2DM model) gene deletion and pharmacological suppression of 12-LO prevented the development of diabetes[47,48]. These findings point towards inhibition of 12-LO being a promising target in both T1DM and T2DM for decreasing insulin resistance, β cell dysfunction and cardiovascular complications.

Histone de-acetylases inhibition: Histone de-acetylases (HDAC) Ⅰ, ⅡA, ⅡB, Ⅲ and Ⅳ are involved in inflam-

matory responses in a variety of conditions including diabetes. HDAC inhibitors cause acetylation of the p65 subunit of NF-κB leading to its inhibition and hence a decrease in the inflammatory response. To date, there are no human data, however, animal data support the role of HDAC inhibition in β cell preservation. Linkage analysis has also revealed that a locus in 6q21, associated with both T1DM and T2DM, lies near HDAC2. Beta cell mass expansion has been observed with HDAC Ⅱ A inhibitors. In streptozotocin (STZ)-induced diabetes, ITF2357 an orally active inhibitor against class I and Ⅱ HDAC, leads to the prevention of diabetes[49].

Sirtuin 1: Sirtuin 1 (Sirt1) is a NAD^+-dependent HDAC class Ⅲ deacetylase. Some of the SIRT1 deacetylation substrates (PGc1a, FoXo, p53, and the p65 subunit of NF-κB (10,41-43 proteins) are central regulators of cellular metabolism, energy expenditure, inflammation and stress response pathways in the cell. These may be an additional target in reducing inflammation. Activation of Sirt1 may have an antiinflammatory role to play in the islets. Sirt1 overexpression prevents NF-κB mediated cytokine-induced β cell damage and its expression has been shown to be reduced in pancreatic islets after cytokine exposure[50]. Nicotinamide mononucleotide, a metabolite that augments sirtuin action, rescues islets from reduced insulin secretion after IL-1β and TNF-α exposure[51].

Identification of the targets of each class of HDAC in human islets under inflammatory conditions will aid in the therapeutic application of this emerging class of agents.

FAT-1 transgene: Long-chain n-3 PUFAs act directly by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism indirectly by altering the expression of inflammatory genes through effects on transcription factor activation. In addition, they increase anti-inflammatory mediators such as resolvins. Thus, n-3 PUFAs are potent anti-inflammatory agents. The FAT-1 transgenic mouse, which expresses the Caenorhabditis elegans *FAT-1* gene encoding an n-3 fatty acid desaturase that converts n-6 to n-3 fatty acids (which is absent in mammals) showed augmented production of n-3 polyunsaturated fatty acids. This has been shown to be protective against the development of diabetes after multiple low dose STZ injections, and displays lower levels of IL-1β, TNF-α, NF-κB and 12-HETE[52]. This may be an additional target for inflammation in T2DM.

Recent studies have indicated that ELF5A-1, an ancient and poorly understood protein, is an important regulator of cytokine release and signalling. This protein is the only protein which contains the unique amino acid, hypusine,which is a modified amino acid lysine residue. Hypusine modification by the inhibitory enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase, is required for ELF5A-1 action in cytokine signalling. Therefore, this modification may well be a new therapeutic target for preventing beta cell decline in the setting of diabetes inflammation[53]. Anti-inflammatory therapeutic targets have been used to decrease the conversion from prediabetes to diabetes and the progression of T2DM. Anti-inflammatory therapies have also been used as treatment modalities for the complications of T2DM and are detailed as follows.

Therapeutic treatments targeting inflammatory mediators in diabetic neuropathy

The various proposed mechanisms of diabetic neuropathy include increased reactive oxygen species production, increased protein glycosylation, neurovascular disturbances, and decreased neurotrophic support.Mouse models have shown that NF-κB activation is associated with diabetic neuropathy. Toll-like receptors can also activate NF-κB and lead to increased expression of cytokines and chemokines. The levels of pro-inflammatory cytokines, chemokines and TNF-α have been shown to be increased in mouse and human models, although the pathogenesis is not yet clear. Rodent studies revealed that increased COX-2 expression leads to a decrease in sensory and motor nerve conduction velocities (NCV), endoneurial blood flow, and intraepidermal nerve fiber density in diabetic mice compared to non-diabetic mice. This led to trials of COX-2 inhibitors and other anti-inflammatory drugs in diabetic neuropathy.

Monocytes from T2DM patients demonstrated increased expression of TNF-α, IL-1, IL-6, and IL-8 as compared to healthy controls and T1DM patients; treatment of these monocytes with 1,25-dihydroxyvitamin D3 downregulated the mRNAs of these cytokines[54]. The natural flavonoid, curcumin, led to a dose-dependent decrease in serum TNF-α levels and attenuated thermal hyperalgesia in STZ-treated mice[55,56]. The beneficial effect of this treatment was further enhanced by the use of insulin[57]. Other agents capable of preventing inflammatory-mediated events in rodent models include glicazide and troglitazone both of which attenuate TNF-α levels. Both of these treatments also prevented decreases in myelinated fiber area, fiber density, and the axon/myelin ratio in the tibial nerve of diabetic rats[58,59].

The anti-oxidant, N-acetylcysteine, dose-dependently decreased TNF-α levels[60] which translated into a decreased incidence or severity of neuropathy.

The expression of COX-2 is increased in the peripheral tissues of diabetic neuropathy models. Piroxicam statistically improved STZ-induced decreases in sensory neuron action potential amplitude[61]. The non-selective inhibitors, sulindac and indomethacin, decreased losses in sural and caudal sensory nerve conduction velocity of diabetic rodents compared to control mice[62,63]. Some non-selective COX inhibitors are effective treatment options, and flurbiprofen alone decreased motor NCV (MNCV). In fact, flurbiprofen treatment mimicked STZ-induced changes and did not reverse/alter STZ-induced changes on MNCV[64]. These findings indicate that COX-1 maintains neural function in rodents. Following this observation, studies were planned to assess the efficacy of COX-2 inhibitors. It was found that

celecoxib treatment prevented the decrease in MNCV and sensory nerve conduction velocity (slowing)[65], and meloxicam was shown to protect against MNCV slowing and endoneurial blood flow deficits in diabetic rodents. Intrathecal administration of COX-2 inhibitors led to a dose-dependent attenuation of mechanical behaviour[66]. Selective inhibition of COX-2 *via* pharmacological or gene inactivation played a preventive role in the increased TNF-α expression in the sciatic nerve of STZ-induced diabetic rodents[67]. However, clinical studies with these drugs are lacking. Only one study evaluating NSAID treatment in diabetic patients has been carried out, which demonstrated an improvement in the neuropathy score with ibruprofen and sulindac treatment compared to placebo[68]. However, these results should be interpreted with caution as no healthy age-matched controls were included. The study only compared responders with non-responders. NSAIDS are a double-edged sword in that their long-term use requires caution due to their well-known side effects. Although selective COX-2 inhibitors do not result in gastrointestinal side effects, cardiovascular side effects are a concern, especially in patients with a high risk for cardiovascular disease, of which subjects with DM form a part. However, it is clear that the agents targeting inflammation in diabetic neuropathy are effective only if targeted very early in the course of neuropathy. Evidence demonstrating their effectiveness after the development of diabetic neuropathy in reversing symptoms such as reductions in nerve conduction velocities or nociceptive behaviour is lacking. Larger studies investigating the time course of anti-inflammatory therapeutics should be planned. Current studies have demonstrated no reversal of diabetic neuropathy and the benefits observed only occur after a treatment period of at least 12 wk[69,70]. Overall, more studies are needed to validate these findings.

Therapeutic treatments targeting inflammatory mediators in diabetic retinopathy

Hyperglycemia increases advanced glycation endproduct (AGE) formation, reactive oxygen species and leads to nitric oxide synthatase dysregulation resulting in activation of NF-κB followed by an increase in cytokines (IL-1, IL-6, TNF-α), chemokines such as CCL-2, 58, 10, 12 and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). This leads to activation of endothelial cells, recruitment of inflammatory cells, increased levels of vascular endothelial growth factor (VEGF) and Angiopoietin 2. These factors are involved in the pathogenesis of increased capillary permeability, capillary dropout and neo-vascularization.

The various therapies used as anti inflammatory therapies in diabetic retinopathy hence target VEGF, Angiopoietin 2, various proteinases and chemokines.

The most important factor, which has been extensively investigated in the alteration of the blood retinal barrier (BRB), is VEGF. Levels of VEGF are significantly elevated in patients with diabetic macular edema (DME) as compared to non-diabetic eye diseases[71,72]. VEGF is a potent vasoactive cytokine which increases vascular permeability. The major effect of VEGF is on endothelial tight junction proteins, leading to extravasation of fluid and hence retinal edema. It also induces the phosphorylation of VE-cadherin, occludin, and ZO-1, causing disruption of the barrier[73].

In addition, it also stimulates increased leukostasis in the microvasculature of the retina, which also leads to breakdown of the BRB[74,75].

Therefore, most of the clinical trials on retinopathy have targeted VEGF. Direct VEGF inhibitors include the anti-VEGF aptamer, pegaptanib, the monoclonal antibody fragment, ranibizumab, and the full length antibody bevacizumab. Other drugs include soluble VEGF receptor analogs, VEGF-Trap, small interfering RNAs (siRNAs) bevasiranib, and rapamycin (sirolimus). Some studies have shown that after two years, the mean change in the visual acuity letter score from baseline was 3.7 letters greater in the ranibizumab and prompt laser group, 5.8 letters greater in the ranibizumab and deferred laser group, and 1.5 letters worse in the triamcinolone and prompt laser group[76]. However, it is important that response to the anti-VEGF treatments in DME is variable, and is not as robust as in proliferative diabetic retinopathy or neovascular glaucoma. This implies that the pathogenesis of DME is multifactorial and anti-VEGF therapy is only one player in the overall pathogenesis.

Angiopoietins are another class of inflammatory growth factors that are important modulators of angiogenesis. The levels of angiopoietin-2 (Ang-2) are significantly elevated in patients with clinically significant macular edema[77], indicating that it alters the BRB. In another study increased expression of Ang-2 mRNA and protein has been demonstrated in the retina of diabetic animals[78]. Even in non-diabetic rats, intra-vitreal injection of Ang-2 led to a three-fold increase in retinal vascular permeability. Ang-2 also induces phosphorylation and loss of VE-cadherin[78]. Recent data have suggested that Ang-2 sensitizes endothelial cells to TNF-α-induced ICAM-1 expression and hence monocyte adhesion. This implies that Ang-2 is an autocrine regulator of endothelial cell inflammatory responses. Therefore, Ang-2 plays a permissive role in the augmentation of pro-inflammatory cytokines[79]. This molecule maybe an important therapeutic target in DME. Ang-2 inhibitors in various tumor models have been found to be effective in preventing tumor growth through the modulation of monocyte infiltration and angiogenesis[80]. Matrix metalloproteinases (MMPs) are major regulators of innate and acquired immunity[81]. Knockout mouse models have shown that these molecules play an important role in both acute and chronic inflammation[82]. It has also been shown that MMPs are important for the proteolytic alteration and hence activation of chemokines. They cleave many members of the CCL/monocyte chemoattractant protein (MCP) family of chemokines rendering them proactive,

which amplifies the inflammatory response. Furthermore, MMPs organise the recruitment of leukocytes as an essential component of tumor-associated inflammation[83]. It is now evident that MMPs also play an important role in the pathogenesis of diabetic retinopathy (DR). The vitreous level of proteinases, such as MMP9, are higher in diabetic subjects with DR than without DR[84]. Both MMP2 and MMP9 are elevated in the retina of animal models with early DR[85]. The retinal vascular permeability in diabetic animals is significantly increased which is a result of a decrease in cell-cell junctional protein and VE-cadherin. MMP inhibitors can decrease this vascular permeability[86]. This implies that the proteolytic degradation of VE-cadherin contributes to the BRB breakdown. This is evidence for the role of extracellular proteinases in the alteration of the BRB seen in DR[87]. Hyperglycemia can activate many soluble mediators such as AGE, reactive oxygen species (ROS), and inflammatory cytokines, which can increase MMP levels and activity in the diabetic state. Retinal inflammation leads to increased leukocyte infiltration in the retina, which by binding to endothelial cells activates cellular proteinases such as elastase, followed by removal of VE-cadherin and its associated protein from the cell surface, resulting in alterations in the endothelial monolayer[88]. These studies indicate an important role for these proteinases in DR.

The levels of many chemokines have been shown to be elevated in various studies.The most common chemokine found to be elevated in serum and vitreous is CCL2[89,90]. CCL2, also known as MCP-1, plays an important role in vascular inflammation by inducing leukocyte recruitment and activation. Hyperglycemia increases CCL2/MCP-1 generation in retinal vascular endothelial cells, pigmented epithelial cells and Muller's glial cells[91]. Furthermore, the gene polymorphism of CCL2 has been indicated as a potential risk factor for DR[92].

Studies have shown that genetic knockout of the CCL2 gene in diabetic mice plays a preventive role in alteration of the BRB[93], and that selective inhibition of the CCL2 gene can prevent alteration of the BRB in diabetes. Further studies using selective inhibitors of CCL2 and CCR2 are in progress.

Genistein, a tyrosine kinase inhibitor, has been shown to be effective in reducing diabetes-induced retinal inflammation by interfering with inflammatory signaling (ERK and P38 MAPKs) in activated microglia. This beneficial effect of genistein may represent a new intervention therapy for modulating early pathological pathways long before the occurrence of vision loss in diabetics[94].

Therapeutic treatments targeting inflammatory mediators in diabetic nephropathy

Inflammation activated by the metabolic, biochemical and haemodynamic derangements may play a key role in the development and progression of diabetic nephropathy. Cytokines such as IL-1, IL-6 and TNF-α stimulate the expression of cell adhesion molecules and profibrotic growth factors, increase endothelial permeability, promote mesangial proliferation, glomerular hypertrophy and the production of ROS. Chemokines like Protein kinase C (PKC)-dependent ICAM-1, VCAM-1 and MCP-1 facilitate leukocyte-endothelial adhesion and infiltration into diabetic kidneys. Adiponectin is protective in that it reduces oxidative stress, the production of TNF-α, and leukocyte-endothelial adhesion. Adiponectin has also been shown to interfere with receptor activation of platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Increased mammalian target of rapamycin (mTOR) activity has been shown to cause glomerular hypertrophy and hyperfiltration in diabetic subjects.

Adenosine is a potent autocrine anti-inflammatory and immunosuppressive molecule that is released from cells into the extracellular space at sites of inflammation and tissue injury. The levels of adenosine, an endogenous purine nucleoside, released from various tissues and organs are decreased in diabetic nephropathy (DN)[95]. DN was more severe in A_{2A} receptor knockout mice than in wild-type mice, which suggests that endogenous adenosine may contribute to kidney protection due to diabetes in a similar manner to that in kidney ischemia-reperfusion injury[96]. MCP-1/CCL2 inhibition by propagermanium ameliorated diabetic glomerulosclerosis and is another target for DN[97]. However, clinical inhibitors of CCL2 have shown only partial effects[98]. Even with CCL2 knockout, only a reduction in albuminuria was observed[99].

Pentoxifylline inhibits the expression of TNF-α mRNA levels[100]. In combination with angiotensin-converting enzyme inhibitors and AT1 receptor blockers (ARB), pentoxifylline decreased albuminuria in DN[101,102].

In a prospective, randomized, double-blind, placebo-controlled study, pentoxifylline (1200 mg daily) for 12 mo, in 34 patients with incipient or established DN had a reno-protective effect determined by a significant reduction in urinary albumin excretion in both incipient and established ($P < 0.01$) DN patients. This effect was attributed to a reduction in CRP, IL-6, TNF-α and serum leptin levels ($P < 0.01$)[103].

The results from 7 animal studies and 13 randomized controlled trials on diabetic kidney disease consistently demonstrated that short-term use of pentoxifylline produced a significant reduction in proteinuria and microalbuminuria in patients with diabetic and non-diabetic kidney diseases. The reports on long-term studies also showed that urinary protein excretion was considerably reduced in patients treated with pentoxifylline; however, as these results were mostly based on small clinical trials it is not clear whether the additive anti-proteinuric effect of pentoxifylline is sustained over time. Large scale clinical trials are needed to establish the long-term use of pentoxifylline as a pharmacological alternative for delaying or preventing the development of end-stage renal disease.

Adiponectin has been shown to suppress inflammatory markers including TNF-α, and receptor activation for PDGF, EGF and FGF. Adiponectin has also been shown to preserve nephrin, decrease the expression levels of TGF-β, and reduce albuminuria.

Inhibition of NF-κB in kidney using PPAR-γ[104],

ARB[105], or pentosan polysulfate[106] has been shown to ameliorate DN in animal models. However,the efficacy of inhibition of NF-κB in delaying progression of DN has not been reported.

HMG-CoA reductase inhibitors (statins) have a controversial role in DN. In a subanalysis of the Treating to New Targets study, treatment with 10 mg and 80 mg atorvastatin was found to increase estimated glomerular filtration rate (eGFR)[107], while in the Prevention of Renal and Vascular End-Stage Disease Intervention Trial, treatment with 40 mg pravastatin did not result in an increase in eGFR[108].

The mTOR is a serine/threonine kinase that mediates cell proliferation, survival, size, and mass[109]. Rapamycin decreases hyperglycemia-induced increase in mTOR activity and thus decreases renal changes in DN, including mesangial expansion and glomerular basement thickness[110]. Rapamycin also significantly reduces the influx of monocytes and macrophages associated with the progression of DN[111,112]. It has also been shown to decrease the release of pro-inflammatory cytokines or chemokines including MCP-1, regulate normal T cell expression and secreted, IL-8, and fractalkine[111,112]. Thus, rapamycin represents a new and valuable anti-inflammatory target in DN.

A recent study showed that aspirin decreased albuminuria in patients with DN[113]. In combination with AT1 receptor blockers (ARB) it led to a further decrease in the progression of DN and inflammatory markers compared to when used alone[114]. This effect of COX-2 inhibitors is postulated to occur as a result of the effects on renal hemodynamics and decrease in profibrotic cytokines[115]. However, in another study, treatment with 200 mg/d COX-2 inhibitor for six weeks did not decrease DN[116]. Thus, the overall data for COX-2 inhibitors in DN remains controversial.

PKC is induced by hyperglycemia and insulin resistance. This PKC activation then alters cell signaling molecules including inflammatory cytokines such as NF-κB, IL-6, TNF-α, and plasminogen activator-1 (PAI-1) in endothelial and mesangial cells[117-119]. Ruboxistaurin (RBX), a PKCβ isoform selective inhibitor, has been shown to prevent DN in rodent DN models by inhibiting mediators of extracellular matrix accumulation, TGF-β and amelioration of insulin signalling[120]. Diabetic PKCβ null mice showed decreased albuminuria and mesangial expansion[121]. A phase Ⅱ clinical trial with RBX significantly decreased albuminuria and maintained a stable eGFR[122]. Recently, it was shown that hyperglycemia itself can activate PKCβ isoforms, which increased the detrimental effects of Ang-2 on glomerular endothelial cells and decreased the glucagon-like peptide-1 (GLP-1) receptor, leading to resistance to GLP-1 treatment in DN[123]. Recent findings suggest that hyperglycemia also activates PKCβ and p38 mitogen-activated protein (MAPK) to increase Src homology-2 domain-containing phosphatase-1 and causes VEGF resistance and independent NF-κB activation to induce podocyte apoptosis in DN[124] which may be new targets of treatment.

Exogenous insulin has been shown to inhibit the activation of TNF-α in animal models[125]. Furthermore,insulin inhibits MCP-1 expression and activation of NF-κB in endothelial cells[126]. Recent studies in patients with T2DM have shown that insulin treatment decreases the expression of inflammatory cytokines, such as MCP-1, ICAM-1, soluble VCAM-1 (sVCAM-1), TNF-α, and IL-6[127,128].

Insulin can increase endothelial nitric oxide (NO) production by rapid post-translational mechanisms, mediated by the PI3K/Akt signaling pathway, leading to vasodilatation, an antithrombotic effect, and anti-inflammatory actions[129-131]. Insulin not only stimulates NO production, but also increases the expression of endothelial NO synthase (eNOS)[132]. Recent data indicate that vascular endothelial cell specific insulin receptor knockout mice had decreased eNOS expression in the aorta[133]. Thus, insulin resistance in vascular tissue could contribute to DN. However, to date, the efficacy of exogenous NO donor remains unclear. Insulin and metformin were studied in a trial for 14 wk. Despite substantially improving glucose control, neither insulin nor metformin reduced inflammatory biomarker levels including hsCRP, IL-6, and sTNFR2, which were the main effects evaluated in comparisons between the individual treatment groups (placebo metformin only; placebo metformin and insulin; active metformin only; or active metformin and insulin)[28].

PPARs regulate insulin sensitivity, lipid metabolism, adipogenesis and cell growth[134-137]. Recent studies indicated that a PPAR-γ agonist decreased the expression of inflammatory markers such as PAI-1, ICAM-1, and NF-κB in the kidney in DN and ameliorated renal function[138].

Analysis of the GLP-1 receptor (GLP-1R) has revealed its expression in endothelial cells and kidney[139,140]. In endothelial cells, GLP-1 inhibits the expression of TNF-α and VCAM-1[141]. GLP-1 acts on the glomerular endothelial cells and decreases the signaling pathway of Ang-2 at phospho-c-Raf (Ser338)/phospho-Erk1/2 *via* phospho-c-Raf (Ser259) activated by the cAMP/PKA pathway. Administration of GLP-1 in DN decreases inflammatory markers including PAI-1, CD68, IL-6, TNF-α, NF-κB, and CXCL2 in the kidney[117].

DPP-4 inhibitors provide vascular protection by increasing the bioavailability of GLP-1and its action. They have also been reported to decrease the levels of MCP-1. In addition, they have vasotropic actions and a possible reduction in DN[142]. A recent large phase Ⅲ study showed that linagliptin significantly reduced albuminuria in DN by 30%[143]. However, the role of DPP-4 inhibitors in the regulation of inflammatory cytokines and vasotropic actions remains largely unexplored and open to further trials.

DIABETES, THE METABOLIC SYNDROME AND NON-ALCOHOLIC FATTY LIVER DISEASE

Type 2 diabetes mellitus is part of the metabolic syn-

drome and non-alcoholic fatty liver disease (NAFLD) shares insulin resistance as a common pathophysiology with T2DM. More recently, NAFLD has been proposed, but not yet accepted, as a criterion for defining the metabolic syndrome[144]. Hepatic insulin resistance has a key role to play in the pathogenesis of NAFLD and adiponectin, an abundant adipocytokine, decreases both hepatic and systemic insulin resistance by decreasing inflammation[145]. Hence, adiponectin and its agonists may be promising targets to reduce both hepatic and systemic insulin resistance[146,147]. Exercise, in addition to its benefits in reducing weight and insulin resistance also reduces the levels of inflammatory cytokines implicated in diabetes-associated NAFLD[148]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been used in NAFLD and lead to a significant reduction in the expression of pro-inflammatory molecules (TNF-α and IL-6) and of reactive oxygen species[149]. Inhibition of Bcl-2 (B-cell lymphoma 2), the first member of the Bcl-2 family of apoptosis regulatory proteins encoded by the *Bcl-2* gene, leads to intensification of inflammation in NAFLD[150]. Serum Bcl-2 concentrations in overweight-obese subjects with NAFLD have been shown to be reduced and may represent an additional target for therapy[151]. JNK, insulin resistance and inflammation represent possible links between NAFLD and coronary artery disease. There are few studies on anti-inflammatory drugs such as aspirin, anti-IL-6 receptors, immune-modulators (calcineurin inhibitors), substances which enhance the expression of heat shock proteins (which protect cells from endoplasmic reticulum stress-induced apoptosis), and anti-c-Jun amino-terminal kinases in NAFLD and these require further study[152]. Thus, NAFLD is a chronic low grade inflammation that leads to insulin resistance due to the increased levels of cytokines[153,154], and anti-inflammatory therapies may help decrease the burden of NAFLD and T2DM.

Thus, inflammation has a role to play both in the pathogenesis of diabetes and its complications and it represents a potential target for treatment in both diabetes and its complications.

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P- Reviewer: Bandeira E, Paraskevas KI, Tarantino G
S- Editor: Ji FF **L- Editor**: Webster JR **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (4): Diabetes-related complications

Recent advances on the association of apoptosis in chronic non healing diabetic wound

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Received: August 21, 2014 Revised: October 28, 2014
Accepted: November 7, 2014
Published online: December 15, 2014

Abstract

Generally, wounds are of two categories, such as chronic and acute. Chronic wounds takes time to heal when compared to the acute wounds. Chronic wounds include vasculitis, non healing ulcer, pyoderma gangrenosum, and diseases that cause ischemia. Chronic wounds are rapidly increasing among the elderly population with dysfunctional valves in their lower extremity deep veins, ulcer, neuropathic foot and pressure ulcers. The process of the healing of wounds has several steps with the involvement of immune cells and several other cell types. There are many evidences supporting the hypothesis that apoptosis of immune cells is involved in the wound healing process by ending inflammatory condition. It is also involved in the resolution of various phases of tissue repair. During final steps of wound healing most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass that contains few cells and consists mostly of collagen and other extracellular matrix proteins to provide strength to the healing tissue. This review discusses the various phases of wound healing both in the chronic and acute wounds especially during diabetes mellitus and thus support the hypothesis that the oxidative stress, apoptosis, connexins and other molecules involved in the regulation of chronic wound healing in diabetes mellitus and gives proper understanding of the mechanisms controlling apoptosis and tissue repair during diabetes and may eventually develop therapeutic modalities to fasten the healing process in diabetic patients.

Key words: Apoptosis; Diabetes mellitus; Diabetic foot; Chronic wound; Oxidative stress

Core tip: Uncontrolled diabetes mellitus lead to the chronic non healing wound which further can escort to the Ischemia and coronary artery disease. Reports suggested that the involvement of various mechanisms in the development of chronic non healing wound in patients with diabetes mellitus, among which the oxidative stress plays a pivotal role which then leading to the enhanced apoptosis of lymphocytes, may be playing a critical role in the delay of wound healing. Connexins are gap junction protein and their upregulation during diabetes might be leads to improper gap junction formation attributing to the passage of various, apoptotic and inflammatory signals thereby resulting in delayed healing of chronic diabetic ulcers.

Original sources: Arya AK, Tripathi R, Kumar S, Tripathi K. Recent advances on the association of apoptosis in chronic non healing diabetic wound. *World J Diabetes* 2014; 5(6): 756-762 Available from: URL: http://www.wjgnet.com/1948-9358/full/

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INTRODUCTION

Diabetes mellitus (DM) is a complex, chronic metabolic disorder; affects almost all age group of patients which requires continuous medical care with multifactorial risk reduction strategies beyond glycemic control[1]. Prolonged and uncontrolled DM may leads various complications which is broadly divided into microvascular complications (due to damage to small blood vessels) and macrovascular complications (due to damage to the arteries) affecting several organs, including muscle, skin, heart, brain, and kidneys.

It is reported that patients with DM are increasing rapidly worldwide and it is now recognized that the developing countries like India and China presently face the greatest burden of diabetes. It is the fourth or fifth leading cause of death in most high income countries caused 5.1 million deaths in 2013 and every six seconds a person dies due to diabetes[2]. According to International Diabetes Federation 382 million peoples were diagnosed with diabetes in 2013 which can reach up to 592 million in 2035. Among the countries China and India are having 98.4 and 65.1 million DM patients respectively in 2013 and which could be reach up to 142.7 million in china and 109.0 million in India[2]. Patients with poorly controlled diabetes may be subject to acute complications of diabetes, such as dehydration, poor wound healing, and hyperglycemic hyperosmolar coma.

Patients with DM have 15% higher risk for amputation than the general population due to chronic ulcers. It leads to diabetic neuropathy, which inhibits nociception and the perception of pain[3]. Due to loss of sensation in the feet of DM patients they become unaware of small wounds in the legs and feet, and may consequently fail to prevent infection or repeated injury on time[4]. Further, DM causes immune suppression and damage to small blood vessels, preventing adequate oxygenation of tissue, which can cause chronic wounds[4]. Immune deficiency also takes place in patients with type 2 DM (T2DM) due to the increased apoptosis of lymphocytes[5] and also the increased generation of reactive oxygen species (ROS) in patients with T2DM, might be another factor, which then stimulates downstream apoptotic signalling pathways[6].

In this connection, Desmoulière *et al*[7] reported that the decrease cellularity in wound repair process is achieved by apoptosis of different cell types. It is reported that the reduced rate of apoptosis is correlated with reduced expression of early growth response protein 1 (EGR1) in the 13 d old wound of epidermis of transgenic animal and the EGR1 mediate the proapaptotic signal *via* p53[8] and it clearly vindicated that the induced Egr1 expression plays a critical role in the resolution phase of wound repair by inducing apoptosis in keratinocytes. Further, it is suggested that the Egr1 expression is induced by various proteins among which transforming growth factor beta (TGF-β) is well known[9].

BASIC MECHANISM OF APOPTOSIS

The term "apoptosis" was coined by Kerr *et al*[10] for a morphologically distinct mode of cell death and the other type of cell death is known as necrosis. The key mechanism of apoptosis is endonuclease activation leading to internucleosomal double-stranded chromatin (DNA) fragmentation which occurs in most physiological cell death whereas cell membrane damage takes place in necrosis. Apoptosis is essential, as defects in apoptotic cell death regulation contribute to many diseases including disorders where deregulated cell proliferation occurs (cancer, restenosis) or where cell loss ensues (stroke, heart failure, neurodegeneration, Acquired Immune Deficiency Syndrome)[11]. In wound-healing process apoptosis is responsible for the removal of inflammatory cells and the evolution of granulation tissue into scar tissue[7]. In DM patients delayed wound healing is one of the major problems which are supposed to be takes place due to uncontrolled blood sugar level; it affects apoptosis during the wound healing process[12].

Apoptosis is also known as programmed cell death that may occur in multicellular organisms; leads to characteristic cell changes like blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[13]. It is a complex process which initiates intracellular apoptotic signalling in response to a stress, which may bring about cell suicide. Cell suicide takes place in four separable but overlapping steps; induction, detection, effectors, and removal[14]. The dying cell remnants are removed by phagocytic cells of the macrophage/monocyte lineage. Interestingly, apoptotic bodies may also be engulfed by cells not specialized in phagocytosis (*e.g.*, vascular smooth muscle cells) (Figure 1)[15].

T2DM is associated with elevated level of oxidative stress, which is one of the most important factors responsible for the development of chronic complications of this disease. Antioxidants like reduced glutathione (GSH), superoxide dismutase (SOD) and catalase protects cells against oxidative damages. In our own publication we have shown that oxidative stress is higher in T2DM patients. In T2DM patients with chronic non healing wound, lymphocyte apoptosis is initiated by the augmentation of reactive oxygen species which leads to the increased expression of proapoptotic proteins like Caspases, FAS, BAX and decreased expression of anti-apoptotic proteins like B-cell lymphoma 2 genes (*Bcl-2*) (Figure 2)[6].

In streptozotocin-induced diabetic rats, the elevated blood sugar level increases cellular apoptosis and the least expression of Bcl-2 protein causes deregulation of the wound healing processes (Tables 1 and 2)[16].

The mechanism of apoptosis has been linked with several proteins but two of them are extensively recognised for their regulation in the pathways (Figure 3)[17]:

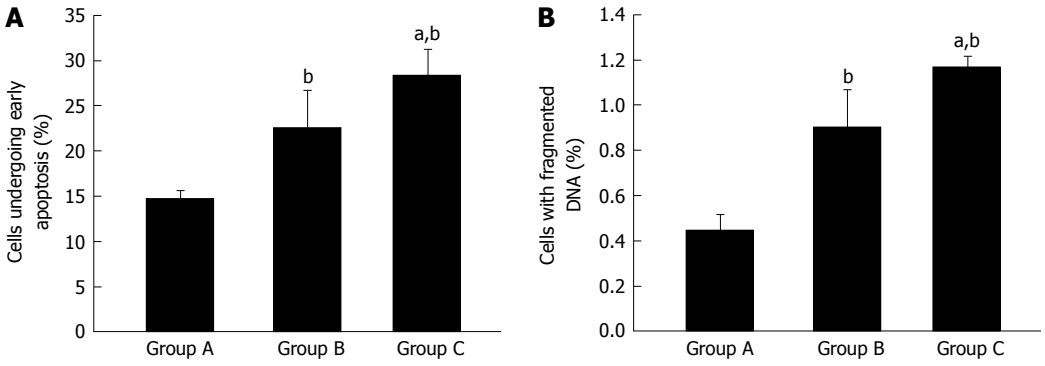

Figure 1 Percentage of apoptotic and dead cells in healthy (Group A), type 2 diabetes mellitus (Group B) and type 2 diabetes mellitus patients with chronic non healing wound (Group C) (A and B). $^{b}P < 0.01$ *vs* healthy; $^{a}P < 0.05$ *vs* uncontrolled diabetes without complication and uncontrolled diabetes with chronic non healing wound. First, second, and third bar in each panel represents healthy, uncontrolled diabetic and uncontrolled diabetic with chronic non healing wound, respectively.

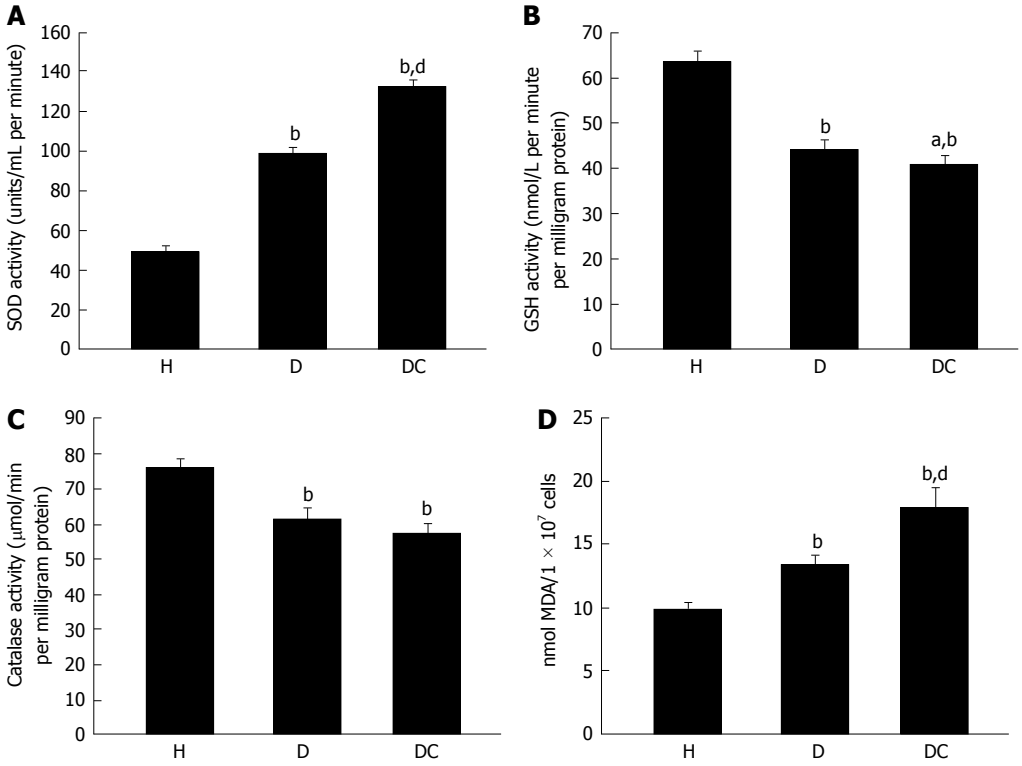

Figure 2 Concentration of superoxide dismutase (A), reduced glutathione (B), catalase (C) and malondialdehyde (D) in healthy (H), type 2 diabetes mellitus (D) and type 2 diabetes mellitus patients with chronic non healing (DC) groups. $^{b}P < 0.01$ *vs* healthy; $^{d}P < 0.01$ and $^{a}P < 0.05$ *vs* uncontrolled diabetes without complication and uncontrolled diabetes with chronic non healing wound. First, second, and third bar in each panel represents healthy, uncontrolled diabetic and uncontrolled diabetic with chronic non healing wound, respectively. SOD: Superoxide dismutase; GSH: Reduced glutathione; MDA: Malondialdehyde.

Table 1 Mean blood glucose level, apoptotic index and DNA fragmentation in control rats (*P* value < 0.01)

	5th day	10th day	20th day	30th day
Control (*n* =10) blood glucose (mg/dL)	75.62 ± 6.41	80.79 ± 11.45	92.05 ± 9.56	90.77 ± 9.7
Apoptotic index (mean ± SD)	1.50 ± 0.60	1.60 ± 0.99	1.64 ± 0.86	1.69 ± 1.12
DNA fragmentation (%) (mean ± SD)	42.25 ± 3.95	44.15 ± 5.61	45.45 ± 5.88	46.58 ± 5.95

(1) targeting mitochondria functionality, or directly transducing the signal *via* adaptor proteins, known as intrinsic pathway; and (2) extrinsic pathway of initiation as identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which can also cause apoptosis *via* calcium binding protease calpain.

In the wound healing process various expression patterns of apoptosis key regulators have been studied

Table 2 Mean blood glucose level, apoptotic index, and DNA fragmentation in rats with diabetes (*P* value < 0.01)

	5th day	10th day	20th day	30th day
With diabetes (n = 10) blood glucose (mg/dL)	467.25 ± 48.2	506.33 ± 35.89	474.99 ± 39.76	488.15 ± 34.36
Apoptotic index (mean ± SD)	3.50 ± 2.60	4.20 ± 2.99	3.60 ± 3.56	3.69 ± 2.75
DNA fragmentation (mean ± SD)	62.80 ± 9.56	74.95 ± 10.45	66.55 ± 8.67	70.48 ± 6.21

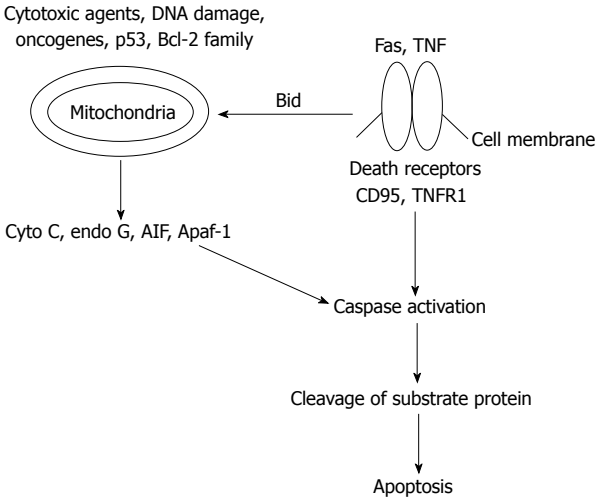

Figure 3 Basic outline of apoptosis mechanism. Bcl-2: B-cell lymphoma 2; TNF: Tumor necrosis factor; AIF: Apoptosis-inducing factor; Apaf-1: Apoptotic protease activating factor-1; TNFR1: Tumor necrosis factor receptor 1.

which shows that the healing in mucosa takes place predominantly through the intrinsic pathway whereas skin healing is predominantly through the extrinsic pathway. The identification of differences in the apoptotic pathways involved in wound healing of various organs may allow the development of therapeutics to improve wound healing[18].

INTRINSIC PATHWAY

The intrinsic signalling pathways involve various arrays of non-receptor-mediated stimuli that produce intracellular signals to work immediately on objects within the cell and are mitochondrial-initiated events. Intrinsic pathway acts both as proapoptotic or antiapoptotic fashion and depends upon the intracellular signals. Negative signals involve the lack of certain growth factors, hormones and cytokines that can escort to collapse of death programs inhibition, thereby triggering apoptosis. Other stimuli that act in encouraging fashion of apoptosis include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals, *etc*.

Stimulus of apoptotic proteins targeting inner membrane of mitochondria may cause mitochondrial swelling through the formation of mitochondrial permeability transition (MPT) pore, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out[19]. Formation of MPT is achieved by the group of proteins consist of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi. The release of cytochrome c into the cytoplasm appears to be a crucial step for the activation of caspase. Once cytochrome c is released it binds with Apoptotic protease activating factor-1 and ATP, which then tie up to pro-caspase-9 to create a protein complex known as apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3. Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting inhibitors of apoptosis proteins activity[20].

In addition to the release of cytochrome c; apoptosis-inducing factor (AIF), endonuclease G and Caspase Activated DNAse (CAD), discharge from the mitochondria during apoptosis. AIF translocates to the nucleus and causes DNA fragmentation into about 50-300 kb pieces and condensation of peripheral nuclear chromatin[21] whereas Endonuclease G translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments[22]. CAD is subsequently discharged from the mitochondria and translocates to the nucleus where after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and chromatin condensation[23]. The control and regulation of these apoptotic mitochondrial events occur through members of the Bcl-2 family of proteins[24]. Bcl-2 proteins are able to promote or inhibit apoptosis by direct action on MAC/MOMPP. Bax and/or Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibits its formation.

EXTRINSIC PATHWAY

The extrinsic signaling pathways involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily[25]. Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain"[26]. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.

TNF-α signaling is linked to the Fas signaling pathway through the interaction of TNF receptor-associated death domain protein with Fas-associated death domain protein and their activation is critically depends upon the activation of caspase[27]. Once caspase-8 is activated, the execution phase of apoptosis is triggered. The binding of three Fas molecules to a Fas ligand (FasL) homotrimer leads to the subsequent binding of Fas-associated death domain and procaspase-8 which finally triggers a cascade of caspase activation, including caspase-3, leading to cell death[28]. Diabetes-enhanced and prolonged expression of

TNF-α and contributes in the direction of impaired healing[29]. TNF-α is found threefold higher in diabetic mouse wounds than wounds in normal mice[30] and threefold higher found in wound fluid from nonhealing venous leg ulcers than in healing ulcers[31].

EXECUTION PATHWAY OF APOPTOSIS

Execution pathways start from the end point of intrinsic and extrinsic pathways of apoptosis. In this phase execution caspase activates to start organized degradation of cellular organelles. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10)[23]. Phagocytic uptake of apoptotic cells is the last component of apoptosis. Mice lacking either of these caspases were deficient in skin wound healing and in liver regeneration[32].

Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the characteristic feature of cell death which can be measured by fluorescent activated cell sorter using annexin V tagged with fluorescent molecule[5].

DIABETIC WOUND HEALING AND APOPTOSIS

Usually wound healing process can be split into 4 temporarily and spatially overlapping phases: coagulation, inflammation, tissue formation (proliferative phase) and tissue remodelling or scar formation phase.

COAGULATION PHASE

Coagulation phase takes place immediately after injury to stop excessive blood flow from wound and provides provisional protection for the wounded area. Hemostatic reaction started with the adherence of platelets to damaged blood vessels giving rise to a blood-clotting cascade. To facilitate aggregation platelates express sticky glycoproteins on their cell membrane[33]. Platelets also released cytokines and growth factors which are a potent chemotactic agent; stimulates the deposition of extracellular membrane to the wound site[34]. In addition, platelets release proinflammatory factors like serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine to dilate blood vessel and increase cell proliferation and migration to the wound area[35].

INFLAMMATORY PHASE

Inflammatory phase starts with the release of platelet-derived growth factor and TGF-A1 and TGF-2 from platelet which attract inflammatory cells, such as leukocytes, neutrophils, and macrophages[36]. Leukocytes release ROS that are antimicrobial and proteases that clear the wound of foreign bodies and bacteria. T lymphocytes playing central role in the wound healing[37] and its increased apoptosis leading to delayed wound healing in diabetic patients[17]. Neutrophils are important in wound healing as they serve to control infection by eliminating microorganisms. With the control of infections neutrophils also release harmful enzymes which damage healthy tissues surrounding the wound site. To prevent further inflammation neutrophils are engulfed by macrophages during the process of apoptosis[38]. Macrophages are the key scavengers for resolving inflammation and facilitating tissue regrowth[39]. These findings show that apoptosis of immune cells could be the major key to end inflammation and initiate healing[40].

Diabetes impaired wound healing by reducing macrophage number and activation which results in the reduced lymphatic vessel formation[41]. The anti proliferative protein p53 involved in apoptosis of inflammatory cells during the healing process and its expression during the healing of cutaneous wounds in swine has been reported by Antoniades *et al*[42].

PROLIFERATIVE PHASE

Proliferative phase of repair begins with the settling down of inflammatory phase and formation of granulation tissue. Granulation tissue formation takes place by growth factors which are released by basal keratinocytes, remaining inflammatory cells and migrating epidermal and dermal cells to support the epithelialization process of wound healing[36]. Diabetes mellitus affects re-epithelialization by affecting multiple proteins and genes including angiopoietin-4[43]. ANGPTL4 shows a potential effect on lipid homeostasis, glucose metabolism, re-epithelialization, inflammation, and potential effect on energy homoeostasis, which is required for wound healing. In corneal wound healing; apoptosis of stromal keratinocyte is well characterised. It triggers subsequent cellular processes that include bone marrow-derived cell infiltration, proliferation, and migration of residual keratinocyte cells and in some circumstances, generation of myofibroblast cells[44].

Diabetes mellitus affects signalling intermediates responsible for coordinating/regulating wound healing angiogenesis and vasculogenesis[45]. Due to the deficiencies in either endothelial progenitor cell or peripheral tissue homing and engraftment of bone marrow, diabetic patients are prone to the development of chronic wounds[46].

TISSUE REMODELING

Tissue remodeling is the process of reformation or restoration of existing tissues. Restoration of a normal blood supply offers an encouraging microenvironment for epidermal and dermal cell migration and proliferation. Fibroblasts proliferate within the wound and synthesize extra-cellular matrix (ECM) forming granulation tissue perfused with newly formed blood vessels.

Wound contraction and matrix remodeling occurs

after the substitution of ECM from collagen Ⅲ, fibrin, fibronectin, and hyaluronic acid[36]. Collagen homeostasis is aberrant in the wound of uncontrolled DM patients who suppose to be mediated by Hsp47; leading to the dysfunction of fibroblast cells. Such impairments could contribute to delayed wound healing[47]. With wound maturation, different cell populations need to be eliminated. Apoptosis of fibroblastic cells occurs, leading to the formation of a relatively acellular scar tissue whose tensile strength is equivalent with unwounded skin. Early studies suggest that endothelial cells undergo apoptosis followed by the removal of myofibroblasts[48].

The passage of various apoptotic and inflammatory signals *via* gap junctions play an important role in tissue remodelling during diabetic wound healing. Connexins (Cx), the gap junction proteins, form channels between two adjacent cells and their expression is highly regulated after wound formation at the transcriptional, translational and post translational levels[49]. In diabetic wounds significant increase in the levels of Cx26, Cx30.3, Cx31, Cx31.1, and Cx43 were observed as compared to non-diabetic wounds[50]. An up regulated connexin expression might lead to the improper gap junction formation attributing to the passage of various, apoptotic and inflammatory signals thereby resulting in delayed healing of chronic diabetic ulcers.

CONCLUSION

Diabetes mellitus delayed normal wound healing by various ways like narrowing of the blood vessels due to arteriosclerosis or leading decreased blood flow and oxygen to a wound, loss of sensation in feet and lowering down the efficiency of the immune system. DM is leading various complications like macroangiopathy and microangiopathy among which Chronic wounds such as venous ulcers are rapidly increasing. In chronic non healing DM patients various cytokines and chemokines are interacting together to lead various complications, *e.g.*, strong positive association between interleukin-7 and monocyte chemoattractant protein 1 may be a possible cause of developing coronary artery disease in these patients[51]. Dysregulation of apoptosis in response to hyperglycemia is universal, leading to impaired wound healing along with the involvement of other target organs. Contrary to the accepted view that diabetic foot is caused by neuropathy and peripheral vascular disease, it now appears that dysregulated apoptosis is emerging as a major cause of the diabetic foot wound. Recent advances in management of DM and understanding of the molecular and cellular components of apoptosis involved during the wound healing phases may enable personalized diagnosis and therapy tailored to a particular patient's needs and therefore lead to better therapeutic outcomes.

ACKNOWLEDGMENTS

Author AK Arya is thankful to Planning and Budgeting Committee, Council for Higher Education, Israel.

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P- Reviewer: Liu C, Sanchez R **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Linking uric acid metabolism to diabetic complications

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Received: August 31, 2014 Revised: October 22, 2014
Accepted: November 7, 2014
Published online: December 15, 2014

Abstract

Hyperuricemia have been thought to be caused by the ingestion of large amounts of purines, and prevention or treatment of hyperuricemia has intended to prevent gout. Xanthine dehydrogenase/xanthine oxidase (XDH/XO) is rate-limiting enzyme of uric acid generation, and allopurinol was developed as a uric acid (UA) generation inhibitor in the 1950s and has been routinely used for gout prevention since then. Serum UA levels are an important risk factor of disease progression for various diseases, including those related to lifestyle. Recently, other UA generation inhibitors such as febuxostat and topiroxostat were launched. The emergence of these novel medications has promoted new research in the field. Lifestyle-related diseases, such as metabolic syndrome or type 2 diabetes mellitus, often have a common pathological foundation. As such, hyperuricemia is often present among these patients. Many in vitro and animal studies have implicated inflammation and oxidative stress in UA metabolism and vascular injury because XDH/XO act as one of the major source of reactive oxygen species Many studies on UA levels and associated diseases implicate involvement of UA generation in disease onset and/or progression. Interventional studies for UA generation, not UA excretion revealed XDH/XO can be the therapeutic target for vascular injury and renal dysfunction. In this review, the relationship between UA metabolism and diabetic complications is highlighted.

Key words: Uric acid; Xanthine dehydrogenase/xanthine oxidase; Diabetes mellitus; Diabetic complications; Xanthine oxidase inhibitor; Metabolism

Core tip: Uric acid (UA) is derived from essential metabolism, and UA metabolism is becoming a novel risk and interventional factor of lifestyle-related diseases in this obesity-prone era. The relationship between UA metabolism and diabetic complications is highlighted in this review and supposed molecular mechanisms are mentioned.

Original sources: Kushiyama A, Tanaka K, Hara S, Kawazu S. Linking uric acid metabolism to diabetic complications. *World J Diabetes* 2014; 5(6): 787-795 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/787.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.787

URIC ACID METABOLISM

Gout, which is caused by increased serum uric acid (SUA) levels, is becoming one of the most prevalent lifestyle-related diseases. According to the National Livelihood Survey in Japan, 874000 people go to hospital for gout in 2004. This constitutes an increase of 3.4 times compared with 1986. Higher prevalence of metabolic syndrome (MetS) is one possible cause for this increase in gout cases, as both the reduced excretion and increased production of UA have been suggested to be associated with MetS. Increased visceral adiposity also causes MetS. In mice, evidence exists that UA is secreted from bloated adipocytes[1]. No studies in humans have confirmed this finding yet.

Uric acid (UA) (2,6,8-trihydroxypurine, C5H4N4O3) is a purine derivative. UA metabolism is a type of nucleic acid metabolism metabolizing purine and its derivatives (adenine, and guanine). Phosphorus oxidation of adenine and guanine (resulting in ATP and GTP) and UA production are essential for many physiological functions. For example, high fructose consumption cause hyperuricemia.

FACTORS THAT DEFINE SERUM URIC ACID LEVELS

SUA levels are determined by a balance between UA production and excretion. At present, no method for detecting the UA production rate is available in humans. Instead, UA production are indirectly speculated through SUA level and urine excretion. The rate-limiting step of UA production is an enzymatic reaction of the xanthine dehydrogenase/xanthine oxidase (XDH/XO) enzyme that oxidizes hypoxanthine-xanthine into UA. Human XDH/XO was cloned in 1993 by Richard[2]. It is expressed in the liver and small intestine of XDH/XO-rich parenchyma cells[3] and is thought to be the major source for SUA. The enzyme is also expressed in adipose tissue, the vascular endothelium, and macrophages, all of which are implicated in lifestyle-related diseases[4]. The UA production rate is based on the amount of substrate and/or XO activity. Since the generation of reactive oxygen species (ROS) depends on XO activity, XO is one of the major sources of oxidative stress in cells along with nicotinamide adenine dinucleotide phosphate oxidase, myeloperoxidase, lipoxygenase, and nitric oxide synthase[5].

The kidney is an important regulator of circulating UA levels and is responsible for 60%-70% of total body UA excretion[6]. The remaining UA is secreted into the intestine, followed by bacterial uricolysis[6]. UA excretion in the kidney consists of urate secretion and reabsorption, and earlier research suggests the involvement of hyperfiltration[7]. UA apical transporters [uric acid transporter 1, organic anion transporter 4 (OAT4), OAT10, sodium-coupled monocarboxylate transporters 1/2, and Na^+-dicarboxylate cotransporter (NaDC1)], which are expressed in the nephron lumen are implicated in the reabsorption process. The role of basolateral transporters in proximal tubular cell is not clarified except for glucose transporter type 9 (GLUT9). During the secretion process, UA is transported into proximal tubular cells *via* OAT1/3 and/or NaDC3 and then secreted by human uric acid transporter, Na^+-phosphate cotransporter (NPT), ATP-binding cassette sub-family G member 2 (ABCG2), and/or ATP-binding cassette sub-family C member 4. Ninety percent of UA filtered by the kidney is reabsorbed[6]. In the intestine, ABCG2 is responsible for about 50% of UA efflux[8-10].

There are many studies about genetic variations exhibiting hyperuricemia. Among genes introduced above, variants of GLUT9 (SLC2A9)[11,12], NPT (SLC17A1)[13], ABCG2 (BCRP) variant[14], are well established and proved to be important in hyperuricemia as a result of decreased extra-renal urate excretion. Genome-wide association study is applied for detecting loci affecting serum UA level. Recent report identified 18 new loci (18 new regions in or near TRIM46, INHBB, SFMBT1, TMEM171, VEGFA, BAZ1B, PRKAG2, STC1, HNF4G, A1CF, ATXN2, UBE2Q2, IGF1R, NFAT5, MAF, HLF, ACVR1B-ACVRL1 and B3GNT4) associated UA concentrations[15]. Not only transporters, but also transcriptional factors, signaling receptors, enzymes are involved in serum UA level.

UA LEVELS IN TYPE 2 DIABETES MELLITUS AND METS

Table 1 shows association between life-style related diseases and UA metabolism[16-24]. Distinguishing cause and effect is difficult; some diseases raise SUA level, but UA affect disease onset or progression.

In patients with diabetes, the SUA level is low due to increased urate clearance[20,25]. In these patients, hypouricemia is associated with glycosuria[26], decreased metabolic control, hyperfiltration, and a late onset of disease, while elevated SUA is a feature of hyperinsulinemia or insulin resistance[7]. Type 2 diabetes mellitus (T2DM) is a risk factor for nephrolithiasis and has been associated with UA stones[27]. It has been suggested that patients with UA stones, especially if overweight, should be screened for T2DM or MetS[28]. The rate of obesity is increasing in Asia as well as in Western countries[29], and hyperuricemia will increase in patients with T2DM. Novel class of anti-diabetic agent, sodium glucose cotransporter 2 inhibitor lowers serum uric acid through alteration of uric acid transport activity in renal tubule by increased glycosuria[21,30].

T2DM ONSET AND UA LEVELS

Besides age, race, family history of diabetes, body mass index (BMI), glucose intolerance, and MetS, SUA levels have been suggested to be associated with T2DM risk[31]. If elevated SUA levels play a causal role in T2DM, SUA might also indirectly affect the prevalence of diabetic complications. The diabetogenic action of UA was reported in 1950[32]; however, its physiological mechanism is not yet known. SUA levels affect insulin resistance[19] and show a significant correlation with risk factors for MetS (high BMI, blood pressure, fasting plasma glucose, and triglyceride levels) and low HDL cholesterol values[19,31,33,34]. Moreover, high SUA levels were shown to predict MetS in a Japanese cohort[35]. We previously reported an association between inflammation, macrophage activation, and SUA production *via* XDH/XO activation in an animal model[36]. In summary, a link between SUA and insulin resistance has repeatedly been shown, and UA itself reportedly plays an important role in the exacerbation of insulin resistance[37].

Table 1 Association between life-style related diseases and uric acid metabolism

Diseases/status	SUA level	UA production	Focus 1	UA excresion	Focus 2
T2DM	High/low				
Glucosuria	Low			Up	Glomerulus
Insulin resistance	High			Down	Proximal tubule cell
Use of SGLT2 inhibitor	Low			Up	
Retinopathy		Up	Vitreus		
MetS	High	Up	Adipocyte/liver?	Down	Proximal tubule cell
CKD	High	Up	Vascular endothelial cell/inflammatory cell	Down/up	Kidney/intestine
Hypertension	High	Up			
Atherosclerosis		Up	Vascular endothelial cell/inflammatory cell		
Reperfusion injury		Up	Vascular endothelial cell		
Heart failure		Up	Inflammatory cell		
Fructose intake	High	Up	Liver	Down	
Sodium intake	High			Down	
Thiazide administration	High			Down	Proximal tubule cell

UA: Uric acid; SUA: Serum uric acid; T2DML: Type 2 diabetes mellitus; CKD: Chronic kidney disease; MetS: Metabolic syndrome.

DIABETIC COMPLICATIONS AND UA LEVELS

SUA independently predicted the development of vascular complications, both retinopathy and nephropahy and coronary artery calcification in type 1 diabetes study by Bjornstad *et al*[38]. The following section discusses the relationship between SUA levels and each diabetic complication.

Neuropathy

Diabetic neuropathy is occasionally the initial manifestation of disease in T2DM patients[39]. It leads to chronic pain, numbness, and substantial loss of quality of life. The prevalence of diabetic peripheral neuropathy shows a significant correlation with increased UA levels[40]. Several studies demonstrated that, when controlled for confounding factors such as age, gender, BMI, renal function, and/or diabetic duration, SUA levels were high in patients with diabetic polyneuropathy and sudomotor dysfunction[41-43].

The pathophysiology of diabetic neuropathy is not completely understood, and multiple metabolic imbalances underlie the development of diabetic neuropathy[44]. Hyperglycemia, dyslipidemia, and cardiovascular dysfunction are all independent risk factors for neuropathy. Probable etiologic factors include the polyol pathway, non-enzymatic glycation, free radicals, oxidative stress, and inflammation. Oxidative stress and inflammation are involved in XDH/XO activity. It is therefore speculated that UA generation by XDH/XO plays a role in diabetic neuropathy.

Diabetic retinopathy

The presence of diabetic retinopathy (DR) is associated with visceral fat accumulation and insulin resistance in T2DM patients[45]. An earlier report found no significant difference in UA levels between patients with or without retinopathy[46], but several recent studies showed a significant increase of UA-related metabolites levels in DR compared to T2DM[47]. SUA concentration was shown to be associated with an increased severity of DR over a three-year period in patients with T2DM. Cox regression analysis showed that patients with SUA levels in the third (5.9-6.9 mg/dL) and fourth ($\geq$ 7.0 mg/dL) quartiles had increased hazard ratios for DR when compared with patients with SUA in the first quartile ($<$ 4.9 mg/dL)[48]. Furthermore, vitreous UA and glucose concentrations were higher in proliferative than in non-proliferative DR. Focal UA production in the vitreous is thought to be involved in the pathogenesis and progression of DR[49].

Nephropathy

Shichiri *et al*[50] showed that glomerular hyperfiltration also occurs in non-insulin-dependent diabetes mellitus (NIDDM) and that it lowers SUA levels by increasing the renal clearance of urate during the hyperfiltration phase[50]. They suggested that hypouricemia can predict the future progression of incipient nephropathy in NIDDM[50]. However, other reports have implied that high (and not low) SUA levels define the prognosis of chronic kidney disease (CKD)[51]. SUA is also associated with known risk factors for kidney disease progression[52], including hypertension[53], cardiovascular disease[54-56], and atherosclerosis[55]. SUA is an independent risk factor for CKD, even without diabetes[57].

SUA is known to be associated with disease progression in the early stage of diabetic nephropathy[17,58]. We found that the progression of renal dysfunction in patients with type 2 diabetic overt nephropathy with an SUA concentration of $\geq$ 6.3 mg/dL carries a poor prognosis, even though their SUA range is considered high-normal[59]. Our data shows the association between UA and disease progression is independent of diabetic control in multivariate analysis. Another report provided evidence for a clear dose-response relationship between SUA levels and early glomerular filtration rate (GFR) loss in patients with T1DM. The progression and regression of urinary albumin excretion were not associated with UA levels[60]. These studies show that UA is an in-

dependent risk factor for renal dysfunction, even after adjustments for confounding factors. Furthermore, even high-normal SUA levels accelerated renal dysfunction in T2DM patients[17,59-62].

UA is lowered in diabetes mellitus (DM) due to hyperfiltration[50], but decreased UA excretion during renal dysfunction raises SUA levels. Our previous study showed that UA levels in the patients who doubled Cr in the observation period (Cr doubling group) were higher than in the non-doubling group at the same estimated GFR (eGFR) level, suggesting that UA production was increased in the Cr doubling group[59]. These data suggest that higher levels of UA production are involved in the pathophysiology of nephropathy progression.

Several recent studies have been investigating therapeutic interventions to delay nephropathy progression[63-65]. Allopurinol therapy significantly decreases SUA levels in hyperuricemic patients with mild to moderate CKD. Its use is safe and has been shown to help preserve kidney function when used for a duration of 12 mo[63]. Febuxostat has a higher renoprotective effect than allopurinol, inhibits oxidative stress, has anti-atherogenic activity, reduces blood pressure, and decreases pulse wave velocity and left ventricular mass index, most likely due to a strong SUA lowering effect[65]. In an animal diabetic nephropathy model, allopurinol attenuated transforming growth factor-beta1-induced Smad pathway activation in tubular cells[66].

Diabetic foot

There are a few reports regarding the relationship between diabetic foot and UA levels. One study states that elevated UA levels are a significant and independent risk factor for diabetic foot ulcer in female Chinese patients with T2DM[67].

Macrovascular complication

A relationship between SUA levels and the development of atherosclerotic disease has been suggested[68-70]. Moreover, there is epidemiological evidence of an association between hyperuricemia and mortality in patients undergoing percutaneous coronary intervention or presenting with acute myocardial infarction[71-73]. Our study showed that SUA is an independent risk factor for vascular complications, even when adjusted for several confounders, including eGFR[56].

Macroangiopathy includes stroke, peripheral artery disease, and ischemic heart disease. In stroke, SUA levels are higher in patients with cardiac syndrome X, and elevated SUA levels are associated with carotid atherosclerosis[74]. A U-shaped relationship was shown for this correlation, as both the upper and bottom quintiles of SUA were associated with a higher risk of fatal stroke[75]. Besides, our study, a link between peripheral artery disease and UA has been rarely reported[56].

Several interventional studies have proven the efficacy of hyperuricemia treatments. A randomized controlled study showed that allopurinol prolongs exercise capacity (especially exercise time until ST depression) when a high dose of 600 mg/d of allopurinol was administered to patients with chronic stable angina[76]. Allopurinol treatment also protects the heart from ischemic reperfusion[77], and oxypurinol, an allopurinol derivative, improves the left ventricular ejection fraction (LVEF) in congestive heart failure patients with low LVEF[22]. Despite the numerous aforementioned studies, several studies have indicated that no association between UA and ischemic stroke[78] or heart disease[79] exists.

OXIDATIVE STRESS, ISCHEMIA/REPERFUSION, AND VASCULAR ENDOTHELIAL XDH/XO

UA itself reportedly functions as an anti-oxidant[80]. For example, XDH-null mutant Drosophila melanogaster have increased vulnerability to oxidative stress[81]. Uric acid administration improved endothelial function in the forearm vascular bed of patients with type 1 diabetes and smokers[82]. However, UA synthesis is accompanied by the generation of ROS.

XDH/XO in the vascular endothelium is associated with ischemia reperfusion injury. It has also been suggested that XO inhibitors improve endothelium-dependent vascular relaxation in blood vessels of hyperlipidemic rabbits[83]. XO as the source of ROS in ischemia/reperfusion injury has been discovered 30 years ago[84,85], and this injury is preventable with XO inhibitors[86]. XOR inhibition reverses endothelial dysfunction in heavy smokers[87,88]. XO inhibitors have the potential to act as free radical scavengers. Febuxostat, however, does not have this activity but can improve organ changes induced by ischemia/reperfusion[23].

FAT DIFFERENTIATION, INSULIN RESISTANCE, AND XDH/XO IN FAT CELLS

Adipose tissue has a high xanthine oxidoreductase activity in mice[1], and UA is secreted from adipocytes. XDH/XO is a novel regulator of adipogenesis and peroxisome proliferator-activated receptor gamma (PPARγ) activity and is essential for the regulation of fat accretion[89]. In addition, UA and adipose tissue XOR mRNAs are increased in ob/ob mice, and fat mass is reduced by 50% in $XOR^{-/-}$ mice.

ATHEROSCLEROSIS AND XDH/XO IN MONOCYTES/MACROPHAGES

XDH/XO is localized to CD68 positive macrophages in the pathological state[36,90]. Inhibition of XDH/XO in inflammatory mononuclear phagocytes inhibits the migration of neutrophils during acute lung injury[91]. Through inhibition of XDH/XO activity, cytokine-induced neu-

trophil chemoattractant secretion from mononuclear phagocytes is reduced, and small ubiquitin-like modifier of PPARγ and hypoxia-inducible factor 1α levels are increased[92]. Febuxostat activates mitogen-activated protein kinase phosphatase-1 and inhibits inflammation by lipopolysaccharide stimulation through the inhibition of ROS generation[93]. Tungsten, acting as a xanthine oxidase inhibitor, prevents the development of atherosclerosis in ApoE knockout mice fed a Western-type diet[94].

XDH/XO activity is also important for lipid accumulation[36]. XDH/XO knockdown or allopurinol administration inhibited foam cell formation in macrophage J774.1 cells. The production of inflammatory cytokines associated with foam cell formation was reduced by allopurinol and febuxostat, and these medications also significantly improved calcification and lipid accumulation in the aortic plaque of ApoE-KO mice[36,95]. It should be noted that the expression of XDH/XO and the deposition of UA are seen in macrophages in arteriosclerotic lesions[96]. *In vitro*, febuxostat inhibited cholesterol crystal-induced ROS formation[95].

Some reports describe XDH/XO as an endogenous regulator of cyclooxygenase (Cox)-2[97] in the inflammatory system, and XDH/XO is central to innate immune function[98]. XDH/XO is thought to be upstream of PPARγ in lipid retention[89] and also induces Cox-2 to induce inflammation, forming a potential feedback loop. In our study, administration of allopurinol to J774.1 cells inhibited secretion of inflammatory cytokines such as tumor necrosis factor α, interleukin (IL)-1β, and IL-6[36]. Gout-associated uric acid crystals activate the NALP3 inflammasome[99]. UA crystals can injure organelle such as lysosomes, and damaged organelle selectively sequestered by autophagy[100]. If mitochondria is damaged, autophagosome is driven *via* microtubule to NLRP3 inflammasome[101]. Colchine treatment expresses the anti-inflammatory effect for gout by inhibiting microtubule-driven spatial arrangement, not by inhibiting UA crystallization. Therefore uric acid crystal in inflammatory cells of atherosclerosis lesion might activate inflammation, while solvent uric acid acts as antioxidant. Microtubule-driven spatial arrangement might be a possible target for diabetic complication derived from UA crystals.

SIGNIFICANCE OF FUTURE UA METABOLISM RESEARCH FOR THE TREATMENT OF PATIENTS WITH DIABETES

XDH/XO has been studied for more than a century, and allopurinol has been used before enzyme inhibition therapy was established. In recent years, the various roles of XDH/XO in diverse pathological conditions have been revealed using a wide variety of research techniques, particularly in the field of molecular biology. This progress in research is related to the global demand to target lifestyle-related diseases such as T2DM, coronary artery disease, CKD, and MetS. Novel research has also led to the development of new powerful and safe UA lowering agent.

Obesity rates are increasing rapidly, and consequently, the pathophysiology of T2DM will be increasingly correlated with fat accumulation, chronic inflammation, and oxidative stress. UA metabolism (involving XDH/XO) is thought to play a central role in the pathogenesis of these conditions. Hence, the need for novel research will increase in the future.

CONCLUSION

The incidence of hyperuricemia has been on the increase since decades. The condition seems to be associated with increased insulin resistance and onset and progression of diabetic complications. UA might thus be suitable marker for both risk evaluation and intervention.

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P- Reviewer: Isaka Y **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diabetes mellitus and hypothyroidism: Strange bedfellows or mutual companions?

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Received: July 6, 2014 Revised: October 14, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

Clinicians should be cognizant of the close relationship that exists between two of the most common endocrine disorders, primary hypothyroidism and diabetes mellitus. This applies to patients with both type 1 and type 2 diabetes mellitus (T1DM and T2DM respectively). However, the association is greater in T1DM, probably because of the shared autoimmune predisposition. In patients with T2DM, the relationship is somewhat weaker and the explanation less clear-cut. Factors such as dietary iodine deficiency, metformin-induced thyroid stimulating hormone suppression and poor glycemic control may all be implicated. Further translational research is required for greater clarification. Biochemical screening for abnormal thyroid function in individuals who have diabetes is warranted, particularly in females with T1DM, and therapy with L-thyroxine appropriately instituted if hypothyroidism is confirmed.

Key words: Type 1 diabetes; Type 2 diabetes; Primary hypothyroidism; Autoimmune disorders; Thyroid screening; Thyroid treatment

Core tip: Clinicians should be cognisant of the close relationship that exists between two of the commonest endocrine disorders, primary hypothyroidism and diabetes mellitus. This applies to both type 1 and type 2 diabetes. However the association is greater in type 1 diabetes, probably due to shared autoimmune predisposition. In type 2 diabetes, the connection is more complex. Biochemical screening for thyroid dysfunction in patients with diabetes is advised.

Original sources: Joffe BI, Distiller LA. Diabetes mellitus and hypothyroidism: Strange bedfellows or mutual companions? *World J Diabetes* 2014; 5(6): 901-904 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/901.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.901

INTRODUCTION

Two of the main clinical disorders encountered in endocrine clinics are diabetes mellitus and primary hypothyroidism. Diabetes can be divided into type 1 diabetes mellitus (T1DM), frequently the result of autoimmune islet-cell destruction, and T2DM, whose pathogenesis embraces both environmental and genetic components[1,2]. Primary hypothyroidism, on the other hand, usually follows autoimmune damage to thyroid tissue by circulating antibodies[3]. The concurrence of these two frequently encountered endocrine conditions in a particular patient has aroused much debate[4]. T1DM and primary hypothyroidism both share an autoimmune predisposition, while T2DM and hypothyroidism could be connected by the concurrence of two frequently occurring endocrine disorders.

The purpose of this review was to evaluate the evidence for an association of both T1DM and T2DM with hypothyroidism. The comparative frequencies of hypo-

Table 1 Prevalence of hypothyroidism in patients with type 1 diabetes *n* (%)

Gender	Number of subjects	Prevalence of hypothyroidism
Female	246	76 (30.9)[b]
Male	258	26 (10.1)
Total	504	102 (20.2)

[b]$P < 0.001$ *vs* males.

thyroidism in T1DM and T2DM were also assessed.

TYPE 1 DIABETES AND HYPOTHYROIDISM

Autoimmune thyroid disease is the commonest autoimmune disorder associated with T1DM[5]. This should not be surprising as T1DM and autoimmune thyroid disease share an autoimmune disposition, and recent studies have shown a shared genetic susceptibility to both conditions[6,7]. Regarding the shared genes involved in this immune predisposition, the *CTLA-4*, *HLA* class 11 and *FOXP3* genes have been implicated. Like T1DM, autoimmune thyroid disease is due to organ-specific autoimmunity. There is infiltration of the thyroid gland with T-lymphocytes and the formation of autoreactive antibodies, particularly against thyroglobulin and thyroid peroxidase (TPOAb). These antibodies are commonly found in patients with T1DM and may be present in up to 25% of patients with T1DM at the time of diagnosis of the diabetes[8]. The presence of thyroid antibodies is predictive of the later development of autoimmune thyroid dysfunction, usually hypothyroidism but also, less commonly, hyperthyroidism[9]. Umpierrez *et al*[10] reported that in patients with type 1 diabetes who had been followed for 18 years, those who were TPOAb-positive were much more likely to become hypothyroid than patients who showed negative antibodies at the outset.

Should hypothyroidism occur, even in a subclinical form, it may be associated with increased risk of hypoglycemia, by reduced hepatic glucose output and especially from impaired gluconeogenesis[11]. There may also be reduced linear growth in children and adolescents[12].

The prevalence of hypothyroidism in patients with T1DM has been estimated to be between 17% and 30%[5]. In our own recently published survey of T1DM at a private diabetes clinic in Johannesburg, South Africa[13], we found a 20.2% prevalence of hypothyroidism in 504 patients with established T1DM. Females showed a significantly higher prevalence than did males (30.9% *vs* 10.1%, $P < 0.001$) (Table 1). Our prevalence rate was slightly higher than that in a study by González *et al*[14], which involved smaller patient numbers. That report again emphasized that the presence of thyroperoxidase autoantibodies at T1DM onset was highly predictive for the development of subsequent thyroid dysfunction. In our survey, we also noted an increased prevalence of other organ specific autoimmune diseases such as Addison's disease, celiac disease and pernicious anemia, but at a much lower frequency.

TYPE 2 DIABETES AND HYPOTHYROIDISM

In T2DM, the association with hypothyroidism is more complex. It is unlikely to be a coincidence of two common endocrine disorders, since the prevalence of hypothyroidism is higher than in the general population. This has been demonstrated in a number of epidemiological studies including our own[15-18], with the prevalence of hypothyroidism varying between 11% and over 30% across different ethnic groups, as opposed to 4% reported in the general population[3-19]. The presence of undiagnosed hypothyroidism may increase cardiovascular risk by aggravating dyslipidemia, insulin resistance, obesity and vascular endothelial dysfunction[20,21]. Factors that could be implicated in this association are rather ill defined and may be complex. Insufficient iodine intake in the diet is one possibility, since a recent study highlighted reduced iodine consumption in 3 major American weight reducing programmes[22]. A report documenting a TSH-lowering effect of metformin in T2DM[23] may also be relevant, although the relationship between metformin and hypothyroidism is likely to be a complex one. Our study suggested that metformin usage might actually be protective against hypothyroidism in patients with T2DM or perhaps that suppressed thyroid-stimulating hormone caused by metformin may lead to physicians missing the diagnosis when thyroid-stimulating hormone measurement is the only screening method employed[16]. Additionally, poorly-controlled diabetes may induce alterations in thyroid function tests similar to that occurring in systemic illnesses i.e. lower levels of all thyroid hormone measurements[24]. Finally the possibility of alterations in the gut microflora being detected in both T2DM and thyroid dysfunction warrants attention. Further studies are clearly required to clarify the causal relationships between these two major endocrine disorders.

COMPARATIVE FREQUENCIES OF HYPOTHYROIDISM IN TYPE 1 AND TYPE 2 DIABETES

From our own large database of patients with diabetes in Johannesburg, we were able to establish that the overall frequency of diagnosed hypothyroidism in T1DM was almost double that seen in T2DM (Table 2). This applied to both female and male subjects. The closer association of hypothyroidism with T1DM probably reflects their well-established autoimmune predisposition and confirms the clinical observation that patients with one organ-specific autoimmune condition are at risk of developing other autoimmune diseases[25].

Table 2 Comparative prevalence of hypothyroidism in patients with type 1 and type 2 diabetes *n* (%)

Diabetic subgroup	Number of subjects	Prevalence of hypothyroidism
Type 1	504	102 (20.2%)[b]
Type 2	918	108 (11.8%)

[b]$P < 0.001$ *vs* type 2 diabetes.

RECOMMENDATIONS FOR THYROID SCREENING AND THERAPY

Hypothyroidism can be clinically silent or aspects of poor diabetes metabolic control may mask its clinical features. In view of the extremely high prevalence of hypothyroidism in those with T1DM, screening for thyroid disease should be done in a systematic fashion. Regular screening will unmask a substantial number of individuals with asymptomatic thyroid dysfunction. Current guidelines advise screening type 1 diabetic subjects at the time of diagnosis or initial contact[26,27].

Thereafter, it is recommended that the TSH is measured annually or two-yearly, but more frequently in antibody-positive patients or individuals who develop a goiter[28]. In the event of pregnancy, this becomes a necessity to prevent damage to fetal mental development secondary to undiagnosed maternal hypothyroidism[29].

For patients with T2DM, the recommendations for biochemical screening are less obvious and depend on factors such as sex, ethnic origin and age. Advice regarding routine testing is either vague[27] or firmly against routine yearly screening of type 2 diabetic patients[28]. Gopinath *et al*[30] reported no difference in the 5-year incidence of thyroid dysfunction in elderly patients with and without diabetes and another study by Chubb *et al*[31] also reported no development of frank hypothyroidism in female type 2 diabetes who manifested subclinical disease. This is in contrast to the data presented in this review, which highlights the increased prevalence of hypothyroidism in patients with T2DM. Selective periodic testing of patients with T2DM is probably warranted. Thyroid antibodies and serum thyroid stimulating hormone (TSH) levels are a useful means of identifying patients with diabetes who are at the greatest risk of thyroid dysfunction. Serum TSH concentrations in the upper range of normal appear to predict the development of future hypothyroidism. In one study involving subjects with both T1DM and T2DM, a TSH concentration above 1.53 mU/L predicted later hypothyroidism[32]. Therefore those with TSH concentrations in the upper normal range probably warrant more frequent, perhaps annual, re-testing. Regarding therapy in patients with diabetes, L-thyroxine should be instituted after confirmed biochemical diagnosis. Since patients with T2DM frequently have underlying ischemic heart disease, therapy in these patients should be started at low dosage (*e.g.*, 25 μg daily). This should be gradually increased over time, using the serum TSH level as a marker of adequate replacement. A serum TSH between 0.5 and 2.0 mU/L is generally considered the optimal target range to aim at[33].

CONCLUSION

Diabetes and hypothyroidism are indeed mutual companions based on the clinical studies that we have reviewed. This applies both to patients with T1DM and T2DM, although patients with T1DM are most predisposed. However, in both subtypes of diabetes, females are more vulnerable to develop hypothyroidism. Clinicians should be alerted to the close relationship that exists between these two common endocrine disorders and the importance of biochemical screening for hypothyroidism as indicated above. Appropriate thyroid replacement therapy can be introduced at an early opportunity, when required.

ACKNOWLEDGMENTS

We would like to thank Arlene Fine for her expert secretarial assistance; Michael Brown for his invaluable assistance in editing this manuscript.

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P- Reviewer: Actis GC, Fan Y, Tasci I
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Nonalcoholic steatohepatitis and insulin resistance in children

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Received: August 20, 2014 Revised: October 7, 2014
Accepted: November 7, 2014
Published online: December 15, 2014

Abstract

Various pathological conditions can cause fatty liver in children. Nonalcoholic steatohepatitis (NASH) in children has been known since 1983. However, NASH diagnosed in childhood does not have a favorable outcome. The pathological characteristics of NASH are significantly different between children and adults. Nonalcoholic fatty liver disease (NAFLD)/NASH is accompanied by insulin resistance, which plays a pivotal role in its pathophysiology in both children and adults. In NASH, a "two-hit" model involving triglyceride accumulation (first hit) and liver damage (second hit) has been accepted. Insulin resistance was found to correlate with changes in fat levels; however, it did not correlate with fibrosis or NAFLD activity score in children. Therefore, insulin resistance may be important in the first hit. Because there is obvious familial clustering in NASH, genetic predisposition as well as environmental factors including diet might be the second hit of NAFLD/NASH.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Insulin resistance; Homeostasis model assessment as an index of insulin resistance; Obesity

Core tip: The pathological characteristics of nonalcoholic steatohepatitis (NASH) are significantly different between children and adults. Nonalcoholic fatty liver disease is accompanied by insulin resistance, which plays a pivotal role in its pathophysiology in both adults and children. In NASH, a "two-hit" model involving triglyceride accumulation (first hit) and liver damage (second hit) has been accepted. Insulin resistance was found to correlate with changes in fat levels; however, it did not correlate with fibrosis in children. Insulin resistance may be important in the first hit. Genetic predisposition as well as environmental factors might be the second hit in children.

Original sources: Arata M, Nakajima J, Nishimata S, Nagata T, Kawashima H. Nonalcoholic steatohepatitis and insulin resistance in children. *World J Diabetes* 2014; 5(6): 917-923 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/917.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.917

INTRODUCTION

Fatty liver disease (fatty liver) is a general term for diseases caused by an accumulation of triglyceride (TG) in liver cells. Various pathological conditions such as Turner syndrome, abnormal mitochondrial and fatty acid metabolism, nephrotic syndrome, Down syndrome, and hormonal therapy can cause fatty liver in children. In adults, nonalcoholic fatty liver disease (NAFLD) is defined by fatty liver without obvious causes such as autoimmune hepatitis, viral hepatitis, or drinking history. Histologically, NAFLD is divided into 2 categories: that without (simple steatosis) and that with fibrosis, necrosis, and inflammation [nonalcoholic steatohepatitis (NASH)]. NASH is regarded as a severe form of NAFLD. According to

a population-based study, 4.8% of adults with NAFLD have been reported to develop liver cirrhosis within a mean observation period of 7.6 years[1]. NASH/NAFLD in childhood has been known since 1983[2]. In this review, we introduce the recent findings of pediatric NASH and insulin resistance.

ETIOLOGY

In Japan, 10% of the general population is estimated to have NAFLD, and 1% to have NASH. In adults with obesity and type 2 diabetes insipidus, the rates are higher[3]. A life-table analysis showed a reduction of life expectancy of up to 7 years in adults with obesity[4]. In children, the prevalence of NAFLD/NASH is estimated to be as high as 2.6%-9.6% in the United States and Asian countries, despite significant differences in race and ethnicity[5-7]. Insulin resistance is often accompanied by NAFLD/NASH, and plays a pivotal role in its pathophysiology[8,9]. The prevalence of insulin resistance in obese children foreshadows a worrisome trend for type 2 diabetes. It is estimated that 170 million children under 18 years worldwide are overweight or obese, which is more than 20% of all children in many countries[10]. According to the SERCH for Diabetes in Youth study, more than 20000 individuals below 20 years of age had type 2 diabetes[11]. According to the follow-up study by Feldstein *et al*[12], 4 out of 66 children with NAFLD developed type 2 diabetes 4-11 years after diagnosis. Moreover, during a 20-year follow-up study, 2 children died and 2 underwent liver transplantation for cirrhosis[12].

CLINICAL DIAGNOSIS

There are no specific symptoms associated with NAFLD and NASH in children. However, there is strong fatigability. Furthermore, obesity, sleep apnea, hypertension, hyperinsulinemia, and acanthosis nigricans are often observed. Visceral obesity is a risk factor. Obesity (body mass index of greater than + 2SD) or an increase in weight of 10% or more per year is likely to be present.

Diagnosis of NAFLD and NASH by conventional blood biochemical examination is difficult. Liver biopsy is required for a definitive diagnosis of NAFLD.

For diagnosis, children should be screened for the presence of HBs antigens, HCV antibodies, anti-mitochondrial antibodies, anti-nuclear antibodies, ceruloplasmin, α-antitrypsin, transferrin, *etc*. Approximately 20% of adults with NASH showed positivity for antinuclear antibodies (greater than 160 X)[13]. Similar findings that 7 out of 14 children with NAFLD were positive for antinuclear antibodies or anti-smooth muscle antibodies have been reported by others[14].

In NAFLD, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are usually mildly increased (2-4 times), and the level of ALT is higher than AST[15]. In NAFLD, levels of alkaline phosphatase and γ-glutamyl transferase are occasionally mildly increased. Levels of ALT and AST are higher in NASH than in NAFLD. Patients with cirrhosis show ALT/AST ratios of less than 1.

To differentiate between simple fatty liver and NASH, information on high-sensitivity C-reactive protein levels and insulin resistance [homeostasis model assessment as an index of insulin resistance (HOMA-R) (fasting blood glucose × immunoreactive insulin/405), adipocytokines [tumor necrosis factor (TNF)-α, adiponectin, and leptin], and oxidative stress markers] can be useful[16]. Other markers for NASH such as high levels of serum iron and ferritin, low platelet count, and KICG (same indocyanine green elimination rate constant) and fibrosis markers (hyaluronic acid, type Ⅳ collagen, and procollagen Ⅲ polypeptide) are also used. The NAFIC (NASH, ferritin, insulin, type Ⅳ collagen 7S) score for adults, pediatric NAFLD fibrosis index for children, and enhanced liver fibrosis test are useful to diagnose fibrosis[17].

Matteoni *et al*[18] classified NAFLD into 4 types from pathological findings. Type 1 is simple fatty liver (only fatty liver), type 2 demonstrates steatohepatitis (fatty liver and lobular inflammation), type 3 demonstrates steatonecrosis and ballooning and swelling of hepatocytes, and type 4 demonstrates steatonecrosis and Mallory bodies (liver cell ballooning degeneration) or fibrosis. He also reported the prognosis of each type upon long-term follow-up. Progression to liver cirrhosis or liver-related death were observed in patients with type 3 or 4 NAFLD. There were no cases that progressed to cirrhosis from types 1 and 2. Therefore, types 3 and 4 NAFLD are defined as NASH pathologically[18]. The grading system of necrosis and inflammation and the staging system of fibrosis that was defined by Brunt *et al*[19] are commonly used. On the other hand, NAFLD/NASH demonstrate different characteristics in adults and in children (Table 1)[20]. Figure 1 shows representative liver pathology of adult type and pediatric type NASH.

NAFLD/NASH in most children mainly have the characteristics of fatty changes, inflammation and fibrosis of the portal area, and absence of perisinusoidal fibrosis and hepatocyte ballooning. Patients with strong fibrosis are classified as having type 2 NAFLD/NASH. Schwimmer *et al*[21] classified pediatric NAFLD into 2 types. According to Brunt's pathological classification, the grading of necrosis and inflammation will be very low and staging of fibrosis will be very high in many children. NASH in children requires careful long-term observation.

BASIC PATHOLOGY

The phenotype of NAFLD is metabolic syndrome of the liver, which in general is accompanied by obesity, diabetes mellitus, hyperinsulinemia, and hyperlipidemia. In the onset and progression of insulin resistance and associated obesity, increased free fatty acid (FFA) levels and abnormal adipocytokine secretion are important factors. In NASH, a "two-hit" model involving TG accumulation (first hit) and liver damage (second hit) has been proposed[22].

Deposition of TG in liver cells is determined by the

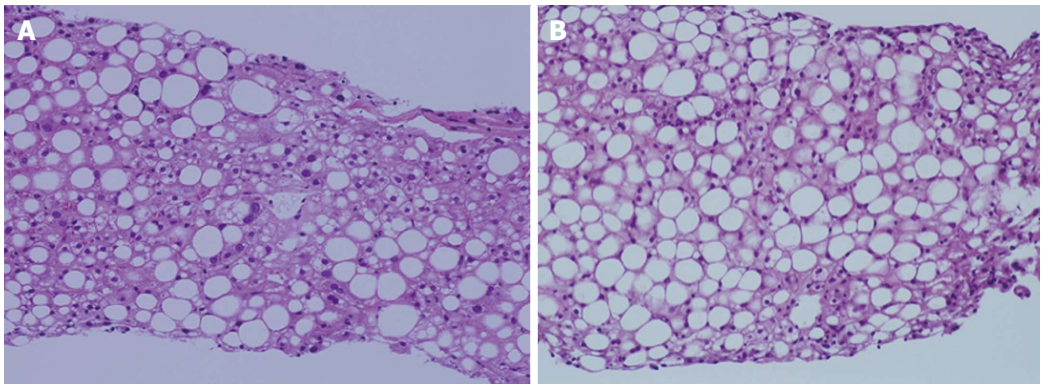

Figure 1 Representative photographs of liver sections of nonalcoholic steatohepatitis/nonalcoholic fatty liver disease patients. A: Pediatric type (type 1) showing severe fibrosis; B: Adult type (type 2) showing mild fibrosis and hepatocyte ballooning.

Table 1 Differences in characteristics of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis between adults and children

	Pediatric-type NASH	Adult-type NASH
Classification by Schwimmer *et al*[21]	Type 2	Type 1
Incidence	Frequent	Rare
Steatosis	Strong Starting in periportal zone (acinar zone 1)	Weak Starting in perivenular zone (acinar zone 3)
Inflammatory cell infiltration	Portal area	Centrolobular area
Hepatocyte ballooning	None	Prevalent
Fibrosis	None or only in periportal zone (acinar zone 1)	Prevalent in perisinusoidal or perivenular zone (acinar zone 3)
Liver cirrhosis	Present	Present
Epidemiology	More common in overweight, colored race (Hispanic: 73%; Asian: 12%), boys > girls	Hispanic: 41%, White, non-Hispanic: 53%, girls > boys
Ratio in pediatric NAFLD (overlap 16%) by Schwimmer *et al*[21]	51%	17%
Ratio in pediatric NAFLD (overlap 50%) by Takahashi *et al*[20]	21%	Not reported

NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

balance of TG-increasing factors (synthesis and influx of TG in liver cells) and TG-decreasing factors (efflux and consumption of TG in liver cells). TG is a molecule composed of 3 fatty acids esterified to a glycerol. Four mechanisms are assumed to affect the level of TGs in the liver cells. The first is increased uptake of FFA from food (15% of TGs in liver) and fatty tissue that supplies the FFA pool in the blood. TG from food is hydrolyzed to FFA by lipoprotein lipase. Non-hydrolyzed TG is supplied to liver cells directly. FFA from fatty tissue in the blood is absorbed by liver cells. Secretion of FFA from adipose tissue is increased when there is insulin resistance. The second is increased FFA synthesis in liver cells (*de novo* synthesis) or reduction of the suppression of FFA synthesis. Fatty acids derived from adipose tissue account for the majority (60%) of hepatic TG accumulation in NAFLD[23]. Nutrients such as carbohydrates, proteins, and lipids are converted to acetyl-CoA and serve as substrates for fatty acid synthesis. The third mechanism is decreased catabolism of FFA in liver cells (consumption by peroxisomes and mitochondrial β-oxidation). The fourth mechanism is decreased release of TG from liver cells (very-low-density lipoprotein is released into the blood by microsomal triglyceride protein)[24]. In children, total parenteral nutrition management, steroid administration, and fatty acid metabolism disorders are representative causes[25]. Oxidative stress, endotoxins, adipocytokines (TNF-α, adiponectin, and leptin) are considered as hepatocyte-damaging factors of the second hit. Hypoxia caused by sleep apnea also has a negative effect.

INSULIN RESISTANCE IN CHILDREN WITH NASH

The effects of steatohepatitis on insulin resistance in children have been elucidated recently. Cali *et al*[26] reported that in children with NASH, there was a significant decrease in insulin sensitivity and impairment in beta-cell function, as indicated by the fall in the disposition index paralleling the severity of hepatic steatosis[26]. Other reports also indicated that the deleterious effects of fat accumulation in the liver affect insulin sensitivity at a multi-organ level[11,27,28]. Consequently, insulin secretion becomes insufficient to maintain glucose levels and some obese children develop beta-cell impairment in the long run. In obese children, beta-cell function has

Table 2 Reports in the literature regarding insulin resistance in pediatric nonalcoholic steatohepatitis /nonalcoholic fatty liver disease

Ref.	Study population and sample size	Age (yr)	Method of diagnosis	Insulin resistance
Santoro *et al*[32]	229 obese children, including 12 cases of liver biopsy-proven NASH	12.8 ± 2.9	MRI and liver biopsy	No significant correlation between MRI-measured steatosis and whole body insulin sensitivity index
Fitzpatrick *et al*[33]	40 liver biopsy-proven NAFLD	10-16	Liver biopsy	68% showed insulin resistance. HOMA-R values did not correlate with NAS
Nobili *et al*[34]	30 NAFLD patients (11:19; without: with steatohepatitis)	8-14	Liver biopsy	HOMA-R values and insulin sensitivity indices did not correlate with steatohepatitis
El-Koofy *et al*[35]	18 patients with normal histology, 8 simple steatosis patients, and 7 NASH patients	2-15	Liver biopsy	HOMA-R values significantly differed between patients with normal histology and those with steatosis/NASH, and significantly correlated with grading based on US
Patton *et al*[36]	88 NAFLD patients	6-17	Liver biopsy	NASH *vs* not NASH: HOMA-R OR = 1.283 (*P*-value = 0.004) and QUICKI OR = 0.786 (*P*-value < 0.001)
Ko *et al*[37]	80 NAFLD patients (18 simple steatosis, 27 type 1 NASH, and 35 type 2 NASH)	10.4 ± 3.9, 12.6 ± 2.4, 12.3 ± 2.3, respectively	Liver biopsy	No differences in HOMA-R values between type 1 and type 2 NASH; HOMA-R values did not correlate with NAS
Manco *et al*[38]	82 NAFLD patients	3-18	Liver biopsy	HOMA-R and QUICKI values, and HOMA-beta secretion did not correlate with NAS
Nobili *et al*[39]	72 NAFLD patients	9-18	Liver biopsy	HOMA-R values did not correlate with NAS, steatosis, inflammation, ballooning, or fibrosis
Chan *et al*[40]	65 fatty liver patients	9.5-14	Liver biopsy and US	HOMA-R and QUICKI values correlated with severity of fatty liver evaluated by US. Higher insulin resistance significantly correlated with fatty liver severity only in male subjects with NASH

NAS: NAFLD activity score; US: Ultrasound; QUICKI: Quantitative insulin sensitivity check index; HOMA-R: Homeostasis model assessment as an index of insulin resistance; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; MRI: Magnetic resonance imaging.

been reported to decrease at a rate of 15% per year[29]. Significant correlations between insulin resistance and NAFLD activity scores (NAS), which were calculated by summing the scores for steatosis, lobular inflammation, and ballooning degeneration, were found in 177 children with NAFLD/NASH[30]. Adipose tissue insulin resistance is also present in the majority of adults with NAFLD, whether the patients are obese or not[31]. Reports in the literature on insulin resistance in pediatric NAFLD/NASH are summarized in Table 2[32-40]. These reports demonstrated that insulin resistance is associated with fatty changes using magnetic resonance imaging and ultrasound[32,40]. However, insulin resistance was not associated with fibrosis or NAS[32-40]. Therefore, these findings suggest that insulin resistance is important for the first hit in the two-hit model of NASH. In adults, insulin resistance did not correlate with NAS but correlated with fibrosis[41,42]. NASH in children is mainly characterized by fatty changes and fibrosis in the portal area (type 2 NASH), which is different to the characteristics of NASH in adults. Therefore, larger scale follow-up studies are required to understand the progression of NASH from children to adults.

CASES OF PEDIATRIC NAFLD/NASH ENCOUNTERED IN OUR DEPARTMENT

Table 3 summarizes the children with NAFLD/NASH that were treated in our department. The patients were 6-16 years old. Their ALT levels were generally high at 16-212 IU/L (normal range < 35 IU/L). Mean values of insulin and HOMA-R values were 23.5 (range: 11.7-272.2 μU/mL and 5.36 (range: 2.07-67.7), respectively. All cases were diagnosed by liver biopsy. All except 1 patient were compatible with type 4 NASH using Matteoni's criteria. The remaining case was type 3. The median NAS was 6 (range: 3-8). The median Brunt's inflammatory grade was 2 (range: 1-3). The median Brunt's fibrosis stage was 3 (range: 1-3). Five cases out of 12 were classified as grade 1, 2 cases were classified as grade 2, and 5 cases were classified as grade 3. The HOMA-R values did not correlate with NAS or Brunt grading.

GENETIC BASIS OF NAFLD/NASH

Familial clustering of NAFLD/NASH is obvious. Genetic predisposition as well as environmental factors including diet have been reported in NAFLD/NASH. Polymorphisms in the genes encoding *PNPLA3*, *UCP3*, *SLC2A1*, *Lipin1*, the *COX-2* promoter, and the *UCP1* (AG + GG) genotypes have been reported to be associated with the development of NAFLD. On the other hand, a genome-wide association study (GWAS) using liver mRNA from NAFLD patients showed that a combination of increased expression of lymphocyte cytosolic protein-1 (*LCP1*) and decreased expression of group-specific component (GC) is significantly associated with susceptibility to NAFLD/NASH. *GC* gene polymor-

Table 3 Pathology and homeostasis model assessment as an index of insulin resistance values of pediatric nonalcoholic steatohepatitis patients treated in our department

Patient number	Age (yr)	Matteoni's criteria	NAS	Brunt's grading	Brunt's staging	HOMA-R
1	6	4	7	3	2	40.6
2	9	4	4	2	2	2.72
3	11	4	6	2	3	4.60
4	11	4	6	2	3	5.83
5	12	4	7	3	3	3.65
6	13	4	5	2	3	58.5
7	14	4	5	2	2	20.0
8	14	4	7	2	2	3.36
9	14	4	8	2	3	3.95
10	14	4	3	1	3	67.7
11	15	4	6	2	2	4.89
12	15	4	7	2	3	17.3
13	16	3	7	2	1	19.4

NAS: Nonalcoholic fatty liver disease activity score; HOMA-R: Homeostasis model assessment as an index of insulin resistance.

Table 4 Efficacy of main drugs against nonalcoholic steatohepatitis/nonalcoholic fatty liver disease symptoms

	Drug	Efficacy
Insulin-sensitizing agent	[1]Metformin[47]	Controversial (effective but no more effective than improvement of lifestyle)
Antioxidants	[1]Vitamin E[47]	Significant improvements in NASH and NAFLD activity scores
	Vitamin C	No changes in ALT levels or liver inflammation; fibrosis was controlled intentionally
Liver-supporting drugs	Ursodeoxycholic acid	No improvements in serum transaminase and fat levels evaluated by US
	Phosphatidylcholine	No improvement in serum ALT level; improvements in liver echo intensity and insulin resistance
	[1]Taurine[48]	Decreased serum ALT levels and increased liver CT values in 7 children
Cholesterol-lowering agents	HMG-CoA reductase inhibitor (atorvastatin)	Decrease in serum ALT levels and improvement in liver pathology
	Probucol	Decrease in serum ALT levels

[1]Indicate drugs reported for children. US: Ultrasound; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; CT: Computed tomography; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; ALT: Alanine aminotransferase.

phisms and LCP1 levels are correlated with vitamin D levels and hyperlipidemia, respectively[43].

Genomic studies on patients with type 2 diabetes revealed some positive correlations of polymorphisms using GWAS. The correlation between gene single nucleotide polymorphisms (SNPs) in *PPAR-gamma*, *TCF7L2*, *G6PC2*, *MTNR1B*, *etc*., have been reported in adolescents as well as in adults[44,45]. In particular, gene SNPs in *TCF7L2*, *IGF2BP2*, *CDKAL1*, *HHEX*, and *HNF1A* might be associated with a higher risk of type 2 diabetes in obese children and adolescents[46]. These genes are involved in the release of insulin granules from beta cells.

MANAGEMENT OF PEDIATRIC NASH AND NAFLD

NAFLD is often associated with obesity, diabetes, hyperlipidemia, and hypertension, and is considered to be a type of metabolic syndrome.

Because NASH is considered to progress from fatty liver, the management of fatty liver is important. Progressive increases in intrahepatic TG levels are associated with progressive impairment of insulin action in skeletal muscle and adipose tissue, in addition to the liver[30]. The principles of treatment are to make improvements in lifestyle, such as diet and exercise. In adults, treatments to improve insulin resistance and oxidative stress have been attempted. The efficacy of insulin sensitizers and antioxidants has also been reported, but there are no established treatments to date.

Quick weight loss can also worsen liver fibrosis. Children with NAFLD often become treatment dropouts, and a relapse is observed in more than 90% of these children. The efficacy of drugs from reports in the literature is shown in Table 4. However, these reports are limited to children[47,48]. In many cases, transaminase levels can be normalized by weight loss of approximately 5%.

The prognosis of NASH in adults is still obscure. Previous studies reported that 5%-20% of patients develop liver cirrhosis within 5-10 follow-up years. Liver re-biopsy within 3-6 years revealed that 40%-50% of patients showed no change, 30%-50% worsened, and 20%-30% improved[49]. AST and ALT levels and disease progression sometimes do not correlate, particularly if there are no subjective symptoms. 10%-20% of the patients showed liver cirrhosis.

A long history of lifestyle-related diseases, severe obesity, type 2 diabetes, low platelet count, rise in fibrosis

markers (hyaluronic acid and type Ⅳ collagen 7S), and liver dysfunction are assumed to affect NASH-associated liver cirrhosis. There are no large-scale studies on childhood NASH, and the prognosis is unknown. Therefore, careful evaluation of fibrosis should be performed during their follow-up.

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P- Reviewer: Barbagallo M, Spinedi E, van den Berg E
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (3): Type 1 diabetes

Distinct clinical and laboratory characteristics of latent autoimmune diabetes in adults in relation to type 1 and type 2 diabetes mellitus

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Received: November 27, 2013 Revised: March 14, 2014
Accepted: June 18, 2014
Published online: August 15, 2014

Abstract

Ever since its first appearance among the multiple forms of diabetes, latent autoimmune diabetes in adults (LADA), has been the focus of endless discussions concerning mainly its existence as a special type of diabetes. In this mini-review, through browsing important peer-reviewed publications, (original articles and reviews), we will attempt to refresh our knowledge regarding LADA hoping to enhance our understanding of this controversial diabetes entity. A unique combination of immunological, clinical and metabolic characteristics has been identified in this group of patients, namely persistent islet cell antibodies, high frequency of thyroid and gastric autoimmunity, DR3 and DR4 human leukocyte antigen haplotypes, progressive loss of beta cells, adult disease onset, normal weight, defective glycaemic control, and without tendency to ketoacidosis. Although anthropomorphic measurements are useful as a first line screening, the detection of C-peptide levels and the presence of glutamic acid decarboxylase (GAD) autoantibodies is undoubtedly the sine qua non condition for a confirmatory LADA diagnosis. In point of fact, GAD autoantibodies are far from being solely a biomarker and the specific role of these autoantibodies in disease pathogenesis is still to be thoroughly studied. Nevertheless, the lack of diagnostic criteria and guidelines still puzzle the physicians, who struggle between early diagnosis and correct timing for insulin treatment.

Key words: Latent autoimmune diabetes in adults; Type 1 diabetes mellitus; Type 2 diabetes mellitus; Autoantibodies; Glutamic acid decarboxylase 65

Core tip: A unique combination of, immunological, clinical and metabolic characteristics has been identified in latent autoimmune diabetes in adults (LADA) patients. Even so, the current definition of LADA fails to capture in one snapshot insulin resistance and autoimmunity, this very special pathognomonic characteristic of LADA. Addressing this dual facet of LADA would undoubtedly provide insight into disease pathogenesis and help in the immediate identification and prompt insulin therapy.

Original sources: Pipi E, Marketou M, Tsirogianni A. Distinct clinical and laboratory characteristics of latent autoimmune diabetes in adults in relation to type 1 and type 2 diabetes mellitus. *World J Diabetes* 2014; 5(4): 505-510 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/505.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.505

INTRODUCTION

As early as in the end-1970s, Irvine identified a group of patients with diabetes who although treated with oral hypoglycaemic agents, they possessed islet cell antibodies

(ICA)[1]. Not only had these ICA-positive patients higher prevalence of other organ specific autoantibodies, they showed a significant tendency to progress faster towards insulin deficiency as well. Interestingly, in these patients persistence of ICA for more than five years from diabetes diagnosis was associated with coexistent of organ specific autoimmune disease and with human leukocyte antigen (HLA)-B8, A1 1. The autoimmune signature in these patients lead to be classified as type 1 diabetes (T1D)[2,3].

Subsequently, a unique combination of immunological, clinical and metabolic characteristics has been identified for this group of patients, namely persistent ICA, high frequency of thyroid and gastric autoimmunity, HLA-DR3 and DR4, progressive loss of beta cells, adult disease onset, normal weight, defective glycaemic control, lower initial levels of C-peptide and impaired response after glucagon stimulation compared to T2D patients, and without tendency to ketoacidosis[4-8]. But, the idea of latent autoimmune diabetes mellitus in adults has been only recently introduced[9]. More specifically, in 1994 Paul Zimmet *et al*[10] and Tuomi *et al*[11] introduce the term latent autoimmune diabetes in adults (LADA) for LADA and 5 years later the 3 criteria that define LADA are suggested, which are (1) GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD) antibody positivity (> 5 RU); (2) age of diabetes onset > 35 years; and (3) insulin independence at diagnosis (at least 6 mo). However, the current definition of LADA fails to capture in one snapshot insulin resistance and autoimmunity, this very special pathognomonic characteristic of LADA[12].

On the other hand, the World Health Organisation diabetes classification does not differentiate LADA as a distinct entity[13]. In fact, the concept of LADA is strongly debated since many researchers question whether LADA is a definite form of diabetes and propose instead that LADA represents slowly evolving T1D which should be regarded as a continuum[14-16]. Even so, LADA can nicely describe patients with features of both T1D and T2D and provide with a better understanding on the grey zone between these two types[17-19]. Addressing this dual facet of their disease would undoubtedly facilitate treatment option and therefore benefit LADA patients.

IMMUNOLOGICAL CHARACTERISTICS

Bottazzo was the first to describe the presence of ICAs in T1D patients having also an endocrine disorder of autoimmune etiology. These antibodies were detected by indirect immunofluorescence on pancreatic cryosections and they were named as such because they targeted unknown elements of islet cells[20].

Nowadays, commercial available kits using pancreas of primate origin are used at routine basis for the determination of ICAs. To facilitate communication among different laboratories and give the possibility of comparable ICA assays, the results should be given in Juvenile Diabetes Foundation units. On the other hand, one should bear in mind that the limitations of ICA assay are the demanding standardization and challenging interpretation of the results. Despite those restrictions, in the 4th International ICA Workshop it was reported that ICA diagnostic test has exceptional specificity and acceptable concordance among the different laboratories[21].

There is a bunch of studies addressing the ICAs relevance to T1D. It is now clear that more than 70% newly diagnosed T1D patients are ICA-seropositive[22]. With a specificity of about 97%, their presence has been reported in less than 4% of healthy subjects[23]. It should be mentioned that in contrast to general population where ICAs higher than 20 JFD is not of clinical relevance, in the first degree T1D relatives this finding is highly prognostic of T1D[24,25]. Finally, it is important for clinicians to closely follow up ICA-positive patients who are receiving oral hypoglycaemic agents, since their presence in this population is strongly predictive of switching to insulin dependency[26].

Anti-insulin autoantibodies (IAAs) were the first specific ICAs to be identified and this was done in 1983 by Palmer *et al*[27] who performed seminal studies in this area using serum from patients who have not been challenged by exogenous insulin at the time of sample collection. Subsequent research have addressed the insulin levels after glucose challenge and it was concluded that insulinopenia was more prevalent in subjects possessing both, ICAs and IAAs, compared to those being positive just for ICAs[28]. However, this marker has a relatively low sensitivity, being even less than 40%[29].

At the 4th International Workshop regarding standardization of IAAs assays it was suggested that RIA should be the method of choice for IAAs determination[30]. However, in the routine laboratory practice their presence can be also assessed by the enzyme-linked immunosorbent assay (ELISA). A reasonable concern would be how the available assays can distinguish between endogenous and exogenous insulin, but this is feasible through distinct idiotypes[31].

Notably, IAAs prevalence is actively influenced by both, sex and age. In detail, in young patients there is an equal incidence of IAAs in both sexes which is skewed at 2 males: 1 female in ages greater than 15 years old[32]. Additionally, these antibodies are inversely correlated with age and since their prevalence sharply drops with age, it is not surprising that they are of low diagnostic value for LADA[33].

The second ICAs specific target to be identified was the GABA-synthesizing enzyme, GAD, a molecule with a size of 64.000 M(r)[34]. Two forms of GAD exist in humans, each transcribed by different gene, termed according to their molecular mass GAD65 and GAD67, and the former being the antigenic target for T1D[35]. Noteworthy, anti-GAD65 autoantibodies are the most typical and prevailing antibodies connected with ICA reactivity[23]. An interesting proposed aspect on anti-GAD65 autoantibodies is that they are present in healthy individuals but they cannot be detected by conventional methods since they are masked by anti-idiotypic antibodies[36].

Anti-GAD65 autoantibodies are detected by commercial available RIAs as well as ELISAs and interestingly enough recent ELISAs offer comparable specificity with RIAs and even better sensitivity[37]. These autoantibodies are positively correlated with age and in the female population are found in greater levels. Serum conversion for these antibodies, from negative to positive, peaks after T1D diagnosis and usually they can be detected even when ICAs becoming gradually undetectable[23]. Since GAD65 is an intracellular antigen, we speculate that during disease progression islet cells could release GAD65, explaining partially the fact that they can be detected after disease onset. For the aforementioned reasons, anti-GAD65 autoantibodies have major role in the management of diabetes in adults. In fact, their positive predictive value in mid-aged population has been reported to be 50%[38].

As regard GAD65 autoantibodies in LADA, the Non-Insulin Requiring Autoimmune Diabetes (NIRAD) nationwide survey has shown that anti-GAD65 titres are useful to categorise patients with adult-onset autoimmune diabetes in two different distinct groups with characteristic clinical picture, autoimmune features, and genetic signature. In detail, patients with higher anti-GAD65 titres can be described by a more profound autoimmunity, quite marked dependency on insulin, higher levels of serum A1C, and lower both body mass index (BMI) and metabolic syndrome prevalence and, regarding genetic traits, decreased frequency of HLA-DRB1*0403 and HLA-DQB1*0602 and an increased for HLA-DRB1*03 and HLA-DQB1*0201 characterises the patients with higher anti-GAD65 titres[39]. Furthermore, studies from the same nationwide survey revealed that in LADA, the variant PTPN22 1858T is strongly associated with high titres of anti-GAD65 autoantibodies while the low levels are correlated to the T2D genetic variant of susceptibility, TCF7L2[40,41]. It has also been suggested that the presence of high anti-GAD65 titres and/or anti-GAD65 autoantibodies directed against the C-terminal and not the middle epitopes of the protein can group a LADA subphenotype with many similarities with classic T1D and a high probability to develop insulin deficiency[42].

On the other hand, other groups do not rely entirely on high anti-GAD65 titres, in order to predict the progression of LADA. Instead, strong predictors are considered the co-existence of positive autoantibodies and both HLA-DRB1 and HLA-DQB1, while, traits including female gender and low BMI and are highly likely to predict insulin requirement within 4 years post-diagnosis[43].

In mid-1990s, two independent groups will add an additional T1D specific autoantigen to the ICAs reactivity panel, the insulinoma antigen 2 (IA-2), a transmembrane molecule belonging to the family of protein tyrosine phosphatases[44-46]. IA-2 is a ubiquitous molecule expressed by neuroendocrine cells, including islet cells of the pancreas, and is localised in the membranes of secretory granules[47].

Within the framework of T1D diagnostic approach, antibodies against IA-2 can be detected by RIA or ELISA commercial kits, with both methods giving comparable results[37]. As a T1D-specific biomarker, anti-IA-2 autoantibodies have a sensitivity of about 60%, meaning that they are less sensitive compared to anti-GAD65 autoantibodies, but when compared to IAA they have higher sensitivity[29]. In contrast to IAA and ICA, anti-IA-2 AAbs show no variation with age and thus, when anti-GAD65 autoantibodies are also evaluated, an autoimmune signature of the diabetes can be defined[23].

Recently, antibodies against the IA-2 (256-760) fragment were shown to be a reliable marker in LADA patients and they were positively correlated with higher frequency of autoimmunity and susceptible HLA haplotypes[48].

Patients with autoimmune diabetes are likely to be presented with an additional autoimmune condition of endocrine (thyroid and adrenal glands) or non-endocrine organs (thyroid and adrenal glands)[23]. Regarding endocrine organ-specific autoimmune conditions, anti-TPO (thyroid peroxidase)/anti-thyroglobulin (anti-Tg) antibodies, marker for autoimmune thyroid disease can be detected in about one fifth of patients with T1D, while anti-adrenal autoantibodies, marker for Addison's disease are rather less common in T1D, being found in less than 2%[49,50]. Regarding non-endocrine organs, autoimmune gastritis, characterised by the presence of anti-parietal-cell antibodies can be found in about one tenth of patients with autoimmune diabetes, while celiac disease, characterised by an immunological signature of anti-endomysial, anti-Tg and anti-gliadin antibodies, with a prevalence of 11% is consider to be common in T1D[50,51].

Regarding organ specific autoantibodies in LADA, the recent NIRAD study 6 suggests a higher frequency of organ-specific antibodies in subjects with high anti-GAD65 titres[52]. They additionally recommend considering that the risk for the presence of other specific antibodies in LADA depends on both, GAD65 titre and gender, and thus, knowledge of the specific odd ratio can be helpful during screening[52].

CLINICAL AND METABOLIC CHARACTERISTICS

First and foremost, the mean age at onset is a highly important hand tool for the clinician, who has to decide upon the different type of diabetes and consequently on the appropriate treatment for the patient as quickly as possible. According to study groups, the age of older than 25 years at onset is a supportive finding towards LADA[19,53]. Furthermore, in comparison to T2D, stimulated as well fasting C-peptide is lower in LADA[5]. Additionally, the level of insulin secretion in LADA is believed to be intermediate between T1D and T2D 5. Importantly, a fast decline in both insulin secretion and stimulated C-peptide secretion occurs rather fast, namely within a few years after LADA diagnosis[54]. In patients over 35 years old at diagnosis and duration of diabetes less than 5 years, the presence of diabetes-specific antibodies is related to lower fasting C-peptide, less often neuropathy

and blood pressure closer to the normal values (56). On the other hand, only patients with more than 1 antibody have reduced residual beta-cell function, and only these patients tend to be leaner[55].

A review by Fourlanos *et al*[54] concludes that patients with LADA are indeed insulin resistant based on homeostasis model assessment, while 50% of insulin secretory failure occurs within the first 4 years. Furthermore, although controversial, in agreement with our observation, LADA patients are presented with lower BMI, blood pressure and triglyceride levels compared to T2D[56].

Regarding treatment policy in LADA patients, time to insulin treatment is based on clinical judgement, with GAD autoantibodies being of upmost importance[57]. Interestingly, Stenström *et al*[58] have suggested that insulin treatment in LADA patients should start as soon as possible. Factually, guidelines on LADA treatment do not exist and is controversial whether sulphonylurea, insulin, vitamin D or alternative therapies such as GAD65, can influence the beta-cell loss progression and metabolic control[59]. Since LADA patients are presented not only with gradually developing insulin deficiency, but also with insulin resistance, a unique treatment strategy should be designed, in order to treat hyperglycaemia and to preserve b-cell function[60].

CONCLUSION

There is adequate evidence that LADA constitutes a special form of diabetes, with a unique immunological, metabolic and clinical signature, while its pathognomonic characteristic can be described as latent autoimmunity, combined with glucose resistance. The lack of a consensus amid diabetes experts hampers the uniformity of the studies and perplexes results interpretation. The need of a clear definition, fulfilling the metabolic and immunologic characteristic of the disease, is unambiguously required. Even better, diagnostic criteria and guidelines would facilitate disease management and pave the way for LADA understanding.

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P- Reviewer: Prasad N, Tamemoto H, Tsilibary PEC
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Chromium does not belong in the diabetes treatment arsenal: Current evidence and future perspectives

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Received: December 11, 2013 Revised: January 23, 2014
Accepted: March 13, 2014
Published online: April 15, 2014

Abstract

Chromium is considered to have positive effects on insulin sensitivity and is marketed as an adjunctive therapy for inducing glucose tolerance in cases of insulin resistance ("the glucose tolerance factor"). Case reports on patients who received prolonged parenteral nutrition indeed showed that the absence of trivalent chromium caused insulin resistance and diabetes. However, whether patients with type 2 diabetes can develop a clinically relevant chromium deficiency is unclear. This review summarizes the available evidence regarding the potential effectiveness of chromium supplementation on glycemic control (Hemoglobin A1c levels) in patients with type 2 diabetes. No studies investigating the long-term safety of chromium in humans were found. All clinical trials that have been performed had a relative short follow-up period. None of the trials investigated whether the patients had risk factors for chromium deficiency. The evidence from randomized trials in patients with type 2 diabetes demonstrated that chromium supplementation does not effectively improve glycemic control. The meta-analyses showed that chromium supplementation did not improve fasting plasma glucose levels. Moreover, there were no clinically relevant chromium effects on body weight in individuals with or without diabetes. Future studies should focus on reliable methods to estimate chromium status to identify patients at risk for pathological alterations in their metabolism associated with chromium deficiency. Given the present data, there is no evidence that supports advising patients with type 2 diabetes to take chromium supplements.

Key words: Chromium; Type 2 diabetes mellitus; Insulin resistance; Therapy; Supplements

Core tip: In some patients who received prolonged parenteral nutrition, absence of trivalent chromium caused insulin resistance and diabetes and supplementation with trivalent chromium "cleared" this metabolic disease. The question is, whether chromium deficiency is a relevant factor in the cause of type 2 diabetes in general and whether supplementation with trivalent chromium can have beneficial effects in type 2 diabetes. Unfortunately, no reliable methods to estimate chromium status exists and according to current evidence, chromium does not improve glycemic control in patients with type 2 diabetes and patients should be advised not to take chromium supplements.

Original sources: Landman GWD, Bilo HJG, Houweling ST, Kleefstra N. Chromium does not belong in the diabetes treatment arsenal: Current evidence and future perspectives. *World J Diabetes* 2014; 5(2): 160-164 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/160.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.160

INTRODUCTION

Insulin resistance is an important target for pharmacological and non-pharmacological interventions in patients with type 2 diabetes. In addition to the well-established interventions, a multitude of suggested alternative solutions outside the field of regular conventional medicine is available. One of these suggested beneficial interventions is oral supplementation with chromium. Chromium is marketed as a substance that improves insulin sensitivity (being as part of the "Glucose Tolerance Factor" molecule), weight loss and improving glycemic control in patients with diabetes[1,2]. Chromium has become the second most popular dietary supplement after calcium in the United States, with sales amounting to approximately 100 million dollars annually[1,2].

Some studies have demonstrated that chromium supplementation in chromium deficient states indeed led to beneficial effects[3-6]. There are strong arguments supporting the hypothesis that chromium supplementation improves glycemic control in chromium deficient patients by improving insulin sensitivity[7]. In addition, patients with diabetes are thought to have a chromium deficient status that is induced by an altered chromium metabolism[4,8,9]. However, other studies have suggested that chromium metabolism is not altered in type 2 diabetes[10]. Unfortunately, cut-off points for chromium levels correlating with relevant changes in glucose metabolism and insulin resistance are lacking. There is no clinically defined chromium deficiency state, nor is there a validated method for estimating the total body chromium status[11-13]. A reliable assessment of the chromium status in biological tissues and fluids is difficult due to extremely low chromium levels[12]. Although some studies have demonstrated successful chromium level determination in hair, sweat, and blood, there is still no exact method for defining chromium deficiency[8]. In this theoretical framework the "diabetic state" is linked to chromium deficiency and chromium supplementation would amend glycemic control by improving insulin sensitivity. This review discusses chromium physiology and summarizes the current evidence that chromium supplementation improves glycemic control in patients with type 2 diabetes.

Several case reports demonstrated beneficial effects of chromium supplementation in patients requiring total parenteral nutrition for prolonged periods[3,5-7,14,15]. One case report, published in 1977, discussed a 40-year-old woman who had undergone a total enterectomy after mesenterial thrombosis and became dependent on total parenteral nutrition[14]. After three years, she started losing weight and developed diabetes mellitus. She was young, had a low body weight, and required 50 IE of insulin daily to reach a near-normoglycemic state. Chromium deficiency was considered as a possible cause. The chromium concentration in her serum and hair was measured and found to be low [154 ng/g (N > 500 ng/g) and 0.55 ng/g (N = 4.9-9.5 ng/g), respectively]. She was treated intravenously with 250 micrograms of chromium chloride daily for two weeks. This treatment decreased the amount of insulin needed, and after four months of chromium supplementation, she remained normoglycemic without insulin. After this and several other case reports[3,9,14], chromium was added to parenteral nutrition as a standard ingredient[6]. Nevertheless, the extent of chromium supplementation necessary during total parenteral nutrition is still debated[16,17].

CHROMIUM PHYSIOLOGY

The two most common forms of chromium are the trivalent (3+) and the hexavalent (6+) forms. Chromium 6+ is not present in nature and is toxic. The chromium found in food and in dietary supplements is the trivalent form. Whole grain products, such as whole grain bread, vegetables, nuts, and some spices contain low concentrations of trivalent chromium. Chromium supplements are available as chromium chloride, chromium nicotinate, chromium picolinate, high-chromium yeast, and chromium citrate. Chromium chloride appears to have a poor bioavailability, although there is limited data on chromium absorption in humans[12,15,18].

The role of trivalent chromium in glucose metabolism has been known since the 1950s[15]. Chromium can alter insulin sensitivity at the cellular level. The oligopeptide Apo-Low-Molecular-Weight-Chromium binding peptide (also known as Apo-chromodulin) plays an important role in potentiating the insulin response in insulin sensitive cells[18,19]. The Apo-chromodulin is loaded intracellularly with a maximum of four chromium ions. Chromium-loaded Apo-chromodulin is called Holo-chromodulin. The Holo-chromodulin molecule binds to the insulin receptor and potentiates the insulin response by activating the receptor. The degree of insulin receptor activation depends on the number of chromium ions bound to this peptide, with a minimum of 0 and a maximum of 4 ions. This chromium binding may lead to an 8-fold difference in insulin receptor activation (when 4 ions are bound compared to 0). Experiments using rat adipocyte cells with equal serum insulin concentrations confirmed that insulin receptor activation is eight times stronger in the presence of chromium than in the absence of chromium[18].

ADVERSE EFFECTS OF CHROMIUM

Several cell culture and animal studies using supraphysiological chromium doses yielded results suggesting that chromium may increase DNA damage[20-23]. Chromium is not unique in this respect; a number of other nutrients such as vitamins A and D, nicotinic acid, and selenium have also been implicated in causing toxicity when taken in excess[24]. Clinical trials of oral chromium supplementation did not demonstrate toxicity in patients on parenteral nutrition[24,25]. We could not find long-term chromium safety studies. The DNA damage identified in cases of supraphysiological trivalent chromium concentrations did not translate into potentially carcinogenic effects when a more physiological dose of oral trivalent chromium was

used in humans[24,26].

CLINICAL EVIDENCE FOR CHROMIUM USE

In 1997, the intervention trial by Anderson *et al*[27] was one of the first chromium-intervention studies in patients with type 2 diabetes. In this randomized controlled trial, chromium picolinate supplements or placebo were administered to 180 Chinese patients with type 2 diabetes. The patients were randomized into three groups: placebo, 200 mg chromium, and 1000 mg of chromium daily. After four months, the hemoglobin A1c (HbA1c) levels in the placebo group were unchanged (8.5%), while they decreased significantly in the 200 mg group, from 8.5% to 7.5%, and decreased in the 1000 mg group, from 8.5% to 6.6%.

In 2007, Balk *et al*[28] performed a systematic review of randomized controlled trials investigating chromium supplementation in patients with type 2 diabetes. At that time, 14 studies with 18 different chromium-based interventions had been performed using HbA1c levels as an endpoint. In 11 out of these 14 trials, there was no significant effect of chromium supplementation. The review by Balk *et al*[28] concluded that, due to the poor quality and heterogeneity of the data, additional studies addressing these limitations were needed before definitive claims could be made about the effect of chromium supplementation[28]. Nevertheless, the meta-analysis by Balk *et al*[28] reported an overall significant effect of chromium supplementation on HbA1c levels (-0.6%; 95%CI: -0.9% to -0.2%). This -0.6% mean benefit was largely due to the inclusion of the data reported by Anderson *et al*[27]. When the Anderson study was excluded, the effect of chromium on HbA1c levels was -0.3% (95%CI: -0.5% to -0.1%; NS)[28]. It should be noted however, that the Anderson study was inadequately blinded with concerns for detection bias and selection bias, and should be considered to be of poor-methodological quality[27,28].

Significant effects in the meta-analysis were only found in studies with poor methodological quality or in studies sponsored by chromium supplement producing companies. In addition, the effects of chromium supplementation were shown to be absent or non-relevant after stratifying the studies according to methodological quality, sponsor involvement, and a western *vs* non-western study location[6,29].

After the review written by Balk *et al*[28], a second Dutch double blind trial was performed in 2008 that studied the effects of chromium on HbA1c levels in patients with type 2 diabetes[29]. After 6 mo, the effect of chromium supplementation compared to placebo on HbA1c levels was 0.24% (95%CI: -0.06% to 0.54%). HbA1c levels were lower in the placebo group compared with the chromium group. All of the trials that have been performed had a relatively short follow-up period. No studies have been performed with sufficient follow-up and the ability to reliably investigate cardiovascular and/or microvascular end-points. All studies used surrogate end-points. None of the trials investigated whether patients had risk factors for chromium deficiency.

Although this review focuses on the most relevant method of estimating glycemic control (HbA1c levels)[30-32], several studies investigated the effect of chromium on other markers of glycemic control[11,13,33-35]. Meta-analyses showed that chromium supplementation did not improve fasting plasma glucose levels[33,36] and had no clinically relevant effect on body weight in individuals with or without diabetes[37-39].

DISCUSSION

Chromium plays a role in insulin physiology, and severe chromium deficiency can lead to insulin resistance. Chromium supplementation may be beneficial in rare cases of prolonged total parental nutrition when standard chromium supplementation is lacking[6]. Despite the lack of sufficient evidence that chromium supplementation improves glycemic control[28,29], chromium is still widely marketed as an effective supplement for improving glycemic control in patients with type 2 diabetes.

Do we need to worry that a low chromium status contributes to hyperglycemia in our patients?

For the average patient with type 2 diabetes, the answer is no. Trivalent chromium is sufficiently available in food, and the occurrence of severe chromium deficiency is highly unlikely. The sparse evidence that chromium supplementation might have effects on glycemic control in a broader population is derived from studies with important methodological flaws[27,28]. Well-performed trials and meta-analyses consistently show that there is no evidence for consistent beneficial effects on glycemic control (as assessed by HbA1c levels) that support prescribing chromium supplements to patients with type 2 diabetes[6,40]. Furthermore, the long-term safety of chromium supplementation has not been established.

Is all hope lost for chromium supplementation in patients with type 2 diabetes?

An important concern when interpreting the data from studies investigating chromium effects is the lack of a validated and precise estimate of chromium status. There is no reliable method for assessing the body's chromium status, and there is no information on the bioavailability of the different forms of chromium[41]. Performing randomized trials in patients with type 2 diabetes will become interesting only when we can properly assess the chromium status in patients at risk for chromium deficiency and when clinically relevant end points are defined.

Recommendations

Future research on chromium should focus on establishing a reliable method for assessing the body's chromium status. The bioavailability of different forms of chromium in Western and non-Western patients should be investigated in order to define a potential effective dose

and to identify patients at risk for chromium deficiency. New randomized trials should only be considered in type 2 diabetes patients with an established chromium deficiency. The long-term safety of chromium supplementation should be investigated in large population studies. Currently, chromium supplementation in patients with type 2 diabetes should not be recommended.

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P- Reviewers: Koya D, Zhao J **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Interrelationships between ghrelin, insulin and glucose homeostasis: Physiological relevance

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Supported by Fonds de Recherche du Québec en Santé (to Laplante M and St-Pierre DH); Operating grants from Natural Sciences and Engineering Research Council of Canada and Diabète Québec; Laplante M also holds operating grants from the Canadian Institutes of Health Research, Canadian Liver Foundation and the Fondation de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec
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Received: December 9, 2013 Revised: February 8, 2014
Accepted: May 8, 2014
Published online: June 15, 2014

Abstract

Ghrelin is a 28 amino acid peptide mainly derived from the oxyntic gland of the stomach. Both acylated (AG) and unacylated (UAG) forms of ghrelin are found in the circulation. Initially, AG was considered as the only bioactive form of ghrelin. However, recent advances indicate that both AG and UAG exert distinct and common effects in organisms. Soon after its discovery, ghrelin was shown to promote appetite and adiposity in animal and human models. In response to these anabolic effects, an impressive number of elements have suggested the influence of ghrelin on the regulation of metabolic functions and the development of obesity-related disorders. However, due to the complexity of its biochemical nature and the physiological processes it governs, some of the effects of ghrelin are still debated in the literature. Evidence suggests that ghrelin influences glucose homeostasis through the modulation of insulin secretion and insulin receptor signaling. On the other hand, insulin was also shown to influence circulating levels of ghrelin. Here, we review the relationship between ghrelin and insulin and we describe the impact of this interaction on the modulation of glucose homeostasis.

Key words: Acylated ghrelin; Unacylated ghrelin; Insulin secretion; β-cell functions; Insulin receptor signalling; Glucose homeostasis

Core tip: The present invited review intends to summarize the current knowledge on the relationships between ghrelin, insulin and glucose homeostasis in cellular, animal and human models.

Original sources: Chabot F, Caron A, Laplante M, St-Pierre DH. Interrelationships between ghrelin, insulin and glucose homeostasis: Physiological relevance. *World J Diabetes* 2014; 5(3): 328-341 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/328.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.328

INTRODUCTION

Obesity and ensuing metabolic complications are major concerns for public health and these disturbances are anticipated to cause the first reduction of life expectancy in modern history[1]. Unfortunately, efforts to curb and especially prevent this alarming trend have so far been met with disappointment. Although it was initially hypothesized that metabolic dysfunctions develop in response to overeating and sedentarity, recent advances show that the

pathophysiological process is much more complex than anticipated. That is, obesogenic environmental and genetic factors disturb homeostatic crosstalk between tissues, promote excessive fat deposition and ultimately alter cellular functions[2-7]. Recently, a close relationship between the development of obesity-related disturbances and gut-derived hormonal dysregulations has been clearly established[8-11]. For instance, studies of gut-derived peptides such as peptide tyrosine-tyrosine 3-36, glucagon-like peptide 1, glucose-dependent insulinotropic peptide and oxyntomodulin have provided key information regarding factors promoting satiety, insulin secretion and glucose disposal. More recently, studies on ghrelin have significantly improved our understanding of mechanisms underlying the stimulation of food intake, lipid accumulation in adipose tissues and the development of metabolic dysfunctions such as insulin resistance and type 2 diabetes[12].

Ghrelin is a 28 amino acid peptide predominantly produced by the stomach[13-15] but also expressed at lower levels in other tissues such as the liver, pancreas, heart, central nervous system (CNS), esophagus and testis[16-18]. Although it was isolated from rat stomach extracts[13] ghrelin was initially shown to induce potent somatotrophic activity in the anterior pituitary[19-21]. Subsequent studies have also revealed the relevance of ghrelin in the regulation of appetite, storage and metabolism of energy substrates, inflammation, stress and other key biological functions[22,23]. Strong evidence indicates the effects of ghrelin in the regulation of metabolic functions and its potential role in the etiology of obesity-related dysfunctions such as insulin resistance and type 2 diabetes[24]. For the purpose of the present work, we will emphasize on reviewing the inter-relationships between ghrelin, insulin and glucose homeostasis.

GHRELIN RECEPTOR

In the circulation, ghrelin is present under acylated (AG) and unacylated (UAG) forms[13]. The enzyme ghrelin o-acyltransferase (GOAT) was shown to be mandatory for the posttranslational addition of the acyl chain on serine-3 of ghrelin[25]. In blood, the half-life of AG is approximately 10 min while UAG displays more stability with a half-life of more than 35 min[26]. Although UAG accounts for approximately 50%-90% of total ghrelin concentrations in the circulation, this form was initially considered as an artifact devoid of biological activity[26,27]. However, recent advances indicate that UAG independently mediates specific biological functions while sharing others with AG.

The effects of AG are mediated through the activation of the native growth hormone (GH) secretagogue receptor 1a (GHS-R1a)[13,28]. Following the discovery of ghrelin, the AG form was reported to stimulate the release of GH and to promote appetite through its action on the brain[13,29-31]. In contrast to its acylated counterpart, UAG was not shown to interact with the GHS-R1a. It has recently been suggested that AG and UAG may exert their effects through the interaction with other receptors than the already identified GHS-R1a. The human ghrelin analog BIM-28163, which fully inhibits GHS-R1a receptor activation induced by native ghrelin, was shown to blunt AG-induced GH secretion[32]. However, since both AG and BIM-28163 induce neuronal activation in the dorsomedial hypothalamus, an important nucleus involved in regulating food intake, it is suggested that an unknown ghrelin receptor could mediate AG's action in promoting weight gain[33,34]. Accordingly, it is proposed that the GHS-R1a acutely mediates AG action on appetite, whereas an unknown ghrelin receptor modulates its chronic peripheral weight-increasing effects[35,36]. It has also been suggested that GHS-R1a could heterodimerize with G protein-coupled receptor 83 (Gpr83)[37]. This study shows that the Gpr83/GHS-R1a dimerization affects ghrelin's ability to activate its only known endogenous receptor, indicating that Gpr83 is an important regulator of ghrelin receptor activity. AG was also shown to interact with several other G protein-coupled receptors such as the dopamine receptor subtypes 1 and 2 (DRD1/2) and melanocortin receptor 3 (MC3R) in the central nervous system[37-41]. Because the existence of another ghrelin receptor remains speculative, the following sections will emphasize on the interactions between GHS-R1a and insulin synthesis/release and signalling.

In a landmark article, Tschöp *et al*[30] had observed that AG increases both food intake and adiposity in rats and mice, suggesting that the hormone promotes positive energy balance. GHS-R1a is predominantly expressed in the central areas known to be influenced by insulin, including hypothalamic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons[42,43]. Furthermore, we and others have reported that the orexigenic effects of AG are mediated through the activation of NPY and AgRP as well as the inhibition of proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons in the arcuate nucleus (ARC) of the hypothalamus[29,44-49]. It has recently been hypothesized that the adipogenic effects of both AG and UAG could be mediated in the CNS by the activation of GHS-R1a[50]. Mice lacking GHS-R1a are protected against early-onset obesity, indicating the importance of ghrelin signaling in regulating body weight[51]. The effect of AG on food intake is believed to be mainly attributable to its interaction with the melanocortin system[44,52]. In fact, in the hypothalamus, ghrelin promotes the expression of the enzyme prolylcarboxypeptidase and therefore the degradation of melanocortin receptor agonist α-melanocyte-stimulating hormone[53]. Central melanocortin signaling has been shown to directly regulate insulin levels and to be independently involved in the control of glucose homeostasis[54]. Moreover, the melanocortin system is an important downstream target for the effects of insulin to regulate food intake and body weight[55]. The melanocortin system is active in areas where both insulin and ghrelin signalling components are expressed; therefore, potential crosstalks between these systems could be envisaged.

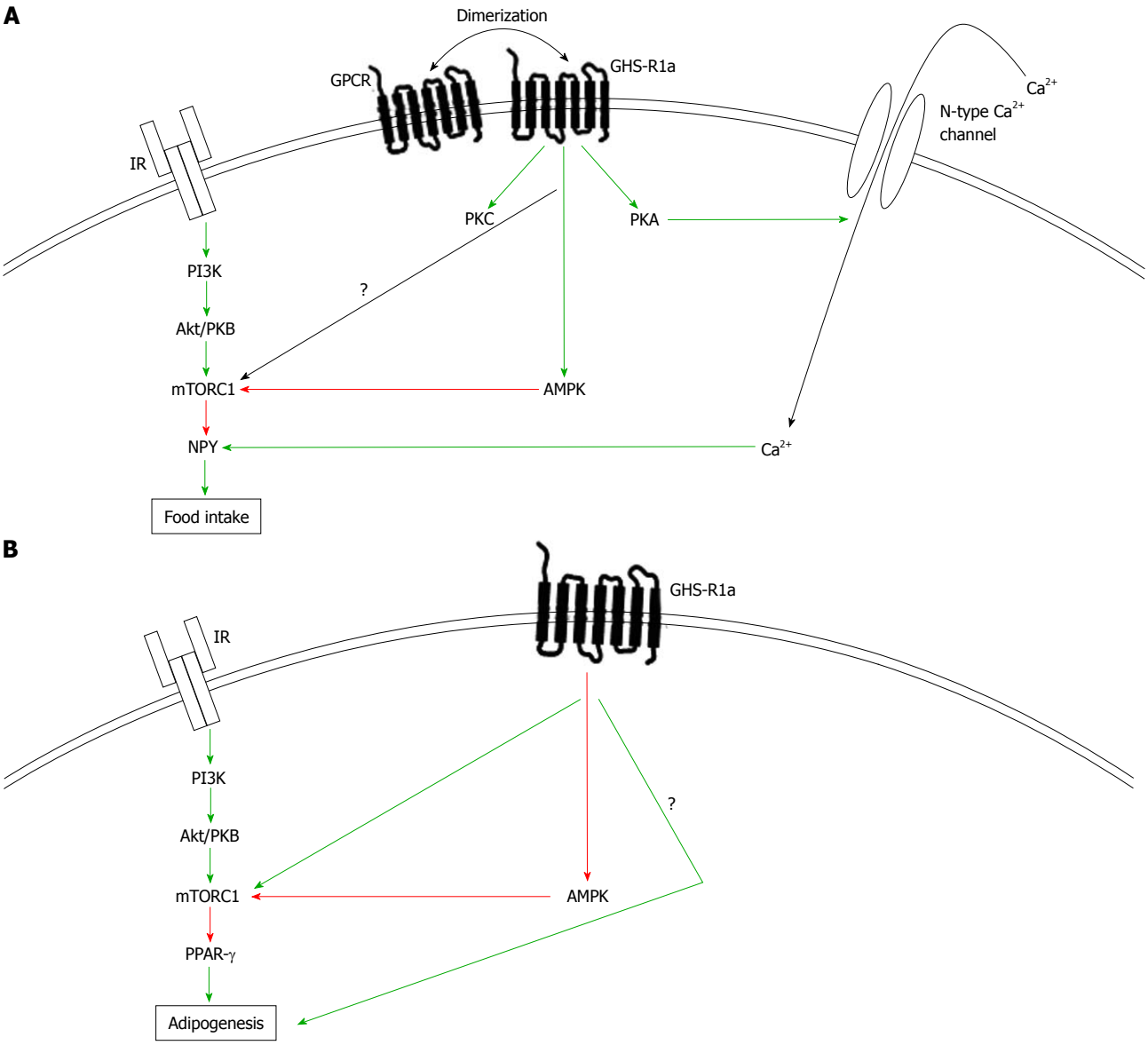

Figure 1 Crosstalks between ghrelin and insulin signaling. A: In the CNS, the interaction between GHS-R1a and ghrelin leads to the activation of PKC and PKA and ultimately to the opening of calcium channels. In the ARC, AG's orexigenic effects are solely mediated through PKA activation and the intracellular entry of Ca^{2+}; which in turn, generate a depolarization/activation of NPY neurons. GHS-R1a activation also triggers AMPK phosphorylation. Also, the activation insulin signaling pathway leads to a phosphorylation cascade that involves PI3K, Akt/PKB and mTORC1. mTORC1 has been shown to reduce food intake by inhibiting NPY expression in ARC neurons. This suggests the existence of a crosstalk between these two signaling pathways, considering that AMPK inhibits mTORC1 activation while ghrelin also reduces the anorexigenic effects of insulin-mTORC1. GHS-R1a could also mediate mTORC1 activation through an AMPK-independent mechanism. Moreover, GHS-R1a has been shown to dimerize with some GPCRs such as Gpr83, DRD1/2 and MC3R; B: In the periphery, the adipogenic effects of ghrelin have been shown to synergize with insulin signaling. In contrast to its central effects, the interaction between GHS-R1a and AG leads to decreases in AMPK activity in the periphery. GHS-R1a also activates Akt, PKB, mTORC1 and ultimately PPAR-γ to stimulate insulin-induced adipogenesis. CNS: Central nervous system; PKC: Protein kinase C; PKA: Protein kinase A; ARC: Arcuate nucleus; GHS-R1a: Growth hormone secretagogue receptor 1a; NPY: Neuropeptide Y; AG: Acylated ghrelin; AMPK: AMP-activated protein kinase; mTORC1: Mechanistic target of rapamycin complex 1; MC3R: Melanocortin receptor 3; DRD1/2: Dopamine receptor subtypes 1 and 2; Gpr83: G protein-coupled receptor 83; GPCR: G protein-coupled receptors; PPAR-γ: Peroxisome proliferator-activated receptor γ; IR: Insulin receptor.

COMMON PATHWAY FOR GHRELIN AND INSULIN RECEPTOR SIGNALING

In the central nervous system

As mentioned above, it is believed that the effects of ghrelin on feeding are mainly exerted through the ARC[29,56,57]. Since the central administration of ghrelin increases the mRNA expression of NPY and AgRP while inhibiting the transcription of POMC and CART, it has been suggested that the orexigenic actions of ghrelin are mediated through the activation of these neurons[29,44-49,58]. As presented in Figure 1A, GHS-R1a activation regulates intracellular calcium through the adenylate cyclase-protein kinase A (PKA) and phospholipase C-protein kinase C (PKC) pathways[43,59]. The PKA pathway has been shown to be related to the orexigenic effects of ghrelin since inhibitors of PKC do not influence the calcium response to ghrelin in NPY neurons of the ARC[43]. Consequently, GHS-R1a activation in the ARC elicits calcium signaling through N-type calcium channel-dependent mechanisms.

AMP-activated protein kinase (AMPK) plays an important role in the regulation of energy metabolism. This kinase is activated following an increase in the AMP/ATP ratio within the cell, a condition linked to cellular energy depletion[60]. Once activated, AMPK phosphorylates acetyl-CoA carboxylase and switches on catabolic processes to promote ATP production[60]. Current evidence indicates that ghrelin could be considered as a signal of energy deficiency since it activates AMPK in the CNS. Moreover, ghrelin-induced calcium entry is substantially suppressed by an AMPK inhibitor[61]. Consistent with these observations, GHS-R1a positively modulates hypothalamic AMPK[61,62]. In turn, the pharmacological activation of AMPK was also shown to stimulate food intake in the hypothalamus[62]. This reinforces the view that AMPK is critical in the control of feeding. However, little is known regarding the potential mechanisms through which AMPK-activation would mediate ghrelin's orexigenic effects. Recent data suggest that in response to fasting, increased ghrelin levels promote feeding through AMPK-mediated activation of hypothalamic fatty acid metabolism in the ventromedial hypothalamus (VMH)[63]. Further studies are needed to identify the mechanisms underlying ghrelin's activation of AMPK and to characterize the neuronal centers involved in the stimulation of appetite.

AMPK influences the insulin signaling pathway, suggesting that ghrelin-induced activation of AMPK could affect this pathway. In fact, the activation of AMPK inhibits the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) activity, a key protein complex activated downstream of the insulin receptor (IR). mTORC1 is a central regulator of cell metabolism, growth, proliferation and survival and acts as a nutrient/hormone sensor[64,65]. In the CNS, mTORC1 activation reduces food intake at least by reducing the hypothalamic expression of NPY and AgRP[66,67]. Recent data indicate that ghrelin requires an intact hypothalamic mTORC1 to stimulate food intake[68]. In this study, the authors suggest that orexigenic effect of ghrelin is mediated by AMPK in the VMH, but through the mTORC1 in the ARC. These results are rather counterintuitive since the effects of AMPK and mTORC1 usually antagonize each other. AMPK activation promotes food intake whereas mTORC1 does the opposite. Indeed, injection of insulin in rodents inhibits AMPK activity in the hypothalamus, promotes mTORC1 activation, and reduces food consumption[69]. Recently, is has been suggested that ghrelin plays a dual time-dependent role in modulating hypothalamus, since it only transiently affects AMPK, which might explain the conflicting results[70]. More studies are needed to better understand the signaling events mediating the effects of ghrelin on the regulation of food intake.

In the periphery

As indicated in Figure 1B, in contrast to its central effects, ghrelin decreases AMPK activity in the periphery, indicating that the hormone bilaterally controls AMPK in the brain and peripherally. Because of this divergence in AMPK activation between the brain and the periphery, it is expected that ghrelin and insulin signaling crosstalks will be different in the CNS versus the periphery. In the periphery, it was observed that ghrelin stimulates adipogenesis[10,22]. The adipogenic effects of ghrelin are mediated, at least in part, through the activation of peroxisome proliferator-activated receptor γ (PPAR-γ), a nuclear receptor whose activity is positively influenced by key components of the insulin pathway, namely Akt/PKB and mTORC1[71-73]. In fact in the periphery, AG promotes adipogenesis through PPAR-γ. Interestingly, a fully operational form of the mTORC1 complex is required for PPAR-γ activation; suggesting that AG's adipogenic effects could be mediated through mTORC1. Consistently, ghrelin promotes activation of the Akt/PKB pathway in macrophages, and this activation results in an enhanced activation of PPAR-γ[74]. Unlike in the CNS, GHS-R1a adipogenic actions seem to synergize with the insulin signaling pathway, establishing the need to further understand the discrepancies between mTOR, AMPK, insulin and ghrelin action in the brain versus peripheral tissues. It is noteworthy that both endogenous and pharmacological activation of AMPK prevent adipogenesis while downregulating the expression of key adipogenic genes including PPAR-γ in the periphery[75,76]. Overall, these elements suggest that ghrelin needs to inhibit peripheral AMPK to exert its effects on fat accumulation.

It is also suggested that the insulin signaling pathway and insulin *per se* can affect ghrelin production and signaling. It has been shown that components of the mTOR signaling pathway are expressed in the endocrine cells of gastric mucosa, where nearly all ghrelin-positive cells are positively stained for these signaling molecules[77]. Moreover, rapamycin, a mTORC1 inhibitor increases gastric ghrelin mRNA, gastric preproghrelin levels and circulating ghrelin, demonstrating that the mTORC1 signaling pathway is crucial in ghrelin expression and secretion[78]. Therefore, insulin could also directly affect ghrelin secretion. Altogether, these findings strongly suggest the existence of a link between ghrelin and insulin signaling pathways. The following sections will focus on the physiological impact of such a relationship on glucose homeostasis, insulin secretion and ghrelin levels in cellular, animal and human models.

GHRELIN AND GLUCOSE HOMEOSTASIS

The influence of ghrelin on the regulation of glucose homeostasis was first hypothesized following the observation of a negative correlation between circulating ghrelin and insulin levels in humans[79]. Later, an association between ghrelin and the homeostasis model of assessment, an index of insulin resistance, in women with polycystic ovary syndrome (PCOS) further supported the involvement of ghrelin in the development of insulin resistance and type 2 diabetes[80]. Subsequently, the association of ghrelin with insulin, glucose and insulin resistance indexes was investigated in different populations with definite metabolic profiles. For instance, in obese and non-obese

children and obese adults with or without insulin resistance or type 2 diabetes, pre-meal total ghrelin levels were inversely associated to insulin levels and the severity of insulin resistance[81-83]. The recent development of new and more sensitive immunoassays has allowed the characterization of distinct biological activity of AG and UAG in healthy and pathological conditions. This led to the observation that AG, rather than UAG, reduces insulin secretion while promoting insulin resistance in individuals with or without metabolic dysfunctions[27,84].

Soon after its discovery, ghrelin was shown to be secreted in a pulsatile manner in response to the nutritional status[31]. In clinical studies, ghrelin levels were initially measured from a unique sample in participants submitted to an overnight fast. However more elaborate study designs have been developed to allow the determination of ghrelin levels at different time points in pre-meal and postprandial conditions. The first evidence suggesting the involvement of ghrelin in the regulation of insulin secretion was provided by the observation of a positive association between suppression of total ghrelin levels and insulin concentrations in the postprandial condition in participants with uncomplicated obesity[85]. In addition, total ghrelin levels were negatively correlated to insulin resistance in obese children and adolescents[83].

As previously reviewed[86,87], several research teams have reported a link between ghrelin and the regulation of glucose homeostasis but this was often achieved using one single fasting sample of total ghrelin. Although they provided key information, data generated from these studies were often not in line with results obtained using AG or UAG treatments in cell, animal and human models. Accordingly, the inverse correlations of ghrelin with insulin levels and insulin resistance commonly described in the literature seem rather counter-intuitive at first glance for an adipogenic hormone promoting food intake and decreased energy expenditure. Indeed, we would expect that ghrelin, which drives food intake and adiposity would be positively associated with impaired metabolic functions. It is therefore likely that under physiological conditions, ghrelin acts as a regulator of energy balance to stimulate appetite and the storage of energy substrates while reducing energy expenditure in periods of limited food availability. However, when nutrients are abundant, ghrelin levels decrease to prevent the excessive accumulation of energy substrates. Some also suggest the existence of a state of ghrelin resistance since high-fat consumption blunts the effects of intracerebroventricular-administrated ghrelin on GH secretion, ARC neurons activation and NPY/AgRP expression[88]. From an evolutionary perspective, ghrelin could favor survival for individuals having limited access to nutrients. However, impairments in the regulation of ghrelin secretion, caused by the ingestion of specific nutrients or other genetic/environmental factors, could promote the excessive accumulation of lipids and ultimately the development of metabolic dysfunctions such as insulin resistance and type 2 diabetes.

EFFECTS OF GHRELIN ON INSULIN SECRETION

It was initially reported that a population of ghrelin- and insulin-producing cells would have common embryonic progenitors within the developing endocrine pancreas[89]. In the pancreas, ghrelin-positive ε-cells are found as single cells in islet periphery. Ghrelin is also co-expressed with glucagon-secreting cells in humans and rats[17,90-94]. The expression of GHS-R1a was also detected in islets as well as in several pancreatic cell lines, suggesting that ghrelin and its receptor could influence pancreatic functions in a paracrine manner[95].

As presented in Table 1, the first direct evidence suggesting the influence of ghrelin on the regulation of insulin secretion was provided by Broglio *et al*[21] in healthy volunteers. In fasting condition, AG administered at 1 μg/kg intravenously (*iv*) significantly reduced circulating insulin levels while increasing glycemia. Using the same conditions, AG was shown to reduce insulin secretion in young and elderly participants[106]. Since AG has a relatively short half-life in circulation, continuous administrations of the peptide were performed to confirm the results obtained using bolus injections. The continuous infusion of AG (1 μg/kg per hour) decreased the first phase of insulin secretion postprandially, while causing a significant rise in glycemia[96,107]. This increase in blood glucose was also associated to an enhanced second-phase insulin response. Similarly, Vestergaard *et al*[101-105] observed that AG infusions (0.3 μg/kg per hour to 1.0 μg/kg per hour) promote insulin resistance; however they did not detect any fluctuation in insulin secretion[100,101]. At lower concentrations (0.3 to 1.5 ng/kg per hour), AG infusions reduced insulin secretion and glucose levels[108]. The same authors have also observed a decrease in insulin secretion in response to the administration of physiological concentrations of AG (0.2 and 0.6 ng/kg per hour)[26,109]. Consequently, it is suggested that physiological levels of ghrelin directly impair β-cell functions but the mechanisms underlying these effects remain to be clarified[109]. One appealing hypothesis is that these inhibitory effects of AG on insulin release could be mediated through the stimulation of somatostatin production[97]. In contrast, a single bolus of AG (1 μg/kg) did not induce any alteration of glucose or insulin levels in obese women[110]. In a clinical study, UAG was administered for 16 h at 1.0 μg/kg per hour and the postprandial insulin response was potentiated in healthy volunteers[111]. Following a meal, the inhibitory effect of AG on insulin release was abrogated by the co-administration with UAG[96]. Furthermore, Kiewiet *et al*[112] reported that the combined treatment with AG and UAG increased insulin sensitivity in morbidly obese patients. Altogether, these studies show that ghrelin has complex effects when administered to humans and that the impact of this hormone on glucose homeostasis likely depends on the dose, the nutritional status and the metabolic profile of the population studied. Furthermore, the biphasic insulin response observed

Table 1 Effects of ghrelin treatment in human participants

Model	Treatment	Dose	Condition	Endogenous insulin	Insulin sensitivity
Healthy or hypopituitary humans	AG *vs* Ctrl (*iv*) AG + Arg *vs* Arg (*iv*)	AG: 1 to 2.2 μg/kg Arg: 0.5 g/kg	Fasting (overnight)	Decreased	Decreased[21,96-99]
Healthy or hypopituitary humans	AG + FFA *vs* FFA AG + UAG	AG: 1 μg/kg FFA: 25 g UAG: 1 μg/kg	Fasting (overnight)	Decreased	No change[96,98]
Healthy humans	AG + OGTT (*iv*) *vs* OGTT UAG *vs* Ctrl (*iv*) AG + UAG *vs* Ctrl (*iv*)	AG: 1 μg/kg OGTT: 100g UAG: 1 μg/kg	Fasting (overnight)	No change	No change[96,98]
Healthy humans	AG *vs* Ctrl (*iv*)	AG: 1 μg/kg	Fasting (overnight)	Increased	Decreased[96]
Healthy humans	AG *vs* Ctrl infusion 3h (*iv*)	AG: 5 pmol/kg per minute	Fasting (overnight)	-	Decreased[100]
Healthy, gastrectomized or hypopituitary humans	EHC: AG *vs* Ctrl 5 h (*iv*) pancreatic clamp + EHC:AG *vs* Ctrl 5 h (*iv*)	AG: 5 pmol/kg per minute	Fasting (overnight)	-	Decreased[101-104]
Healthy humans	EHC: AG 5 h (intramuscular)	AG: non-specified supraphysiological dose	Fasting (overnight)	-	Increased[105]

AG: Acylated ghrelin; *iv*: Intravenous; Arg: Arginine; Ctrl: Control; UAG: Unacylated ghrelin; OGTT: Oral glucose tolerance test; EHC: Euglycemic/hyperinsulinemic clamp.

after the administration of AG indicates that the peptide could exert distinct effects on β-cells: an initial inhibition of insulin release combined to a subsequent stimulation of insulin synthesis[96,107]. Further studies are needed to clarify the causes of the variability in insulin secretion and glucose homeostasis observed in response to ghrelin. To do so, it is critical to establish the concentrations at which ghrelin will be administered, and to design clinical protocols with well-established nutritional status and sufficient blood samples to allow detecting positive/negative effects on insulin release under specific metabolic conditions.

Similarly to the available data in humans, data derived from most rodent studies indicate that AG inhibits insulin secretion. In wild type mice, *iv* administrations of AG (5 nmol to 150 nmol) were shown to inhibit fasting and glucose-induced insulin secretion[113]. In contrast, insulinotropic effects have been reported in response to an *iv* injection of AG (25 nmol/L) in rats[114]. In mice, the administration of AG (1 to 10 nmol/kg, *iv*) was also shown to induce biphasic responses[115]. In fact AG was shown to inhibit insulin release by blocking the effects of a cholinergic antagonist on the activation of phospholipase C (PLC) after 2 min but this effect was reversed 6 min after treatment[115]. During the early phase (2 min), ghrelin also promoted the stimulation of insulin secretion by potentiating the response of the phosphodiesterase inhibitor IBMX, but this effect could no longer be observed at 6 min. The same group also reported that the stimulatory effect of ghrelin on insulin release was accompanied by increases in nitric oxide and that this outcome was mediated by the activation of the neuronal constitutive nitric oxide synthase[116]. In mice, AG promptly inhibits insulin release but this effect is reversed over time. This suggests that AG could block the first-phase of insulin secretion and subsequently allow β-cells to release the hormone. Although these effects were modulated through PLC and phosphodiesterase, the mechanisms underlying these observations remain to be elucidated. Consequently, following the description of this biphasic response, it is even possible to speculate that AG's effects could be mediated through the activation of more than one distinct receptor. For instance, these effects could potentially be regulated by the formation of homo- and heterodimers between GHS-R1a and other receptors such as Gpr83 and DRD1/2[37,41]. Interestingly, the expression of both GHS-R1 and DRD2 was previously reported in β-cells[41,95]. Furthermore, DRD2 was shown to inhibit insulin secretion through the activation of the β_2-adrenergic receptor[117]. This indicates that under distinct conditions, AG (and potentially UAG) could mediate the dimerization of GHS-R1 and consequently exert different effects on β-cell functions.

Genetic manipulations have also provided key data regarding ghrelin actions. Overexpression of the ghrelin (*Ghrl*) gene was shown to decrease insulin levels in mice, while its inactivation was shown to enhance insulin secretion and to prevent glucose intolerance[118-120]. In leptin-deficient mice, the deletion of the *Ghrl* gene potentiates insulin secretion and improves glucose homeostasis[121,122]. The pharmacological inhibition of GHS-R1 was also shown to increase insulin secretion and improve glucose homeostasis[123]. In contrast, the ablation of the *Ghs-r1* gene decreased glucose control and reduced insulin secretion in leptin-deficient mice[124]. This impaired insulin response was associated with the upregulation of Uncoupling protein-2 (Ucp-2), Sterol regulatory-element binding protein-1c (Srebp-1c), Carbohydrate-responsive element-binding protein (Chrebp) and Macrophage migration inhibitory factor-1 (Mif-1) and with the downregulation of Hypoxia-inducible factor-1α (Hif-1α), fibroblast growth factor-21 (Fgf-21) and Pancreatic and duodenal homeobox-1 (Pdx-1) in whole pancreases[124]. These genes are known to decrease (*Ucp-2, Srebp-1c*, *Chrebp* and *Mif-1*) or improve (*Hif-1α*, *Fgf-21* and *Pdx-1*) β-cell functions. Another group has also suggested that the effect of AG could be mediated through an increased production of the β-cell autoantigen for type 1 diabetes (IA-2β)[125]. In perfused rat pancreases, the influence of AG on insulin release was also investigated. AG (10 nmol/L) was shown

to promptly decrease insulin *in situ* secretion[126].

The effects of ghrelin on the regulation of insulin secretion were also investigated *in vitro*. In pancreatic tissue fragments of normal and diabetic rats, treatments with AG (1 pmol/L to 1 μmol/L) induced insulinotropic effects[127]. This effect was also observed in response to high doses of AG (0.1 to 1 μmol/L) in cultured isolated mice islets[115]. In contrast, AG was shown to inhibit insulin secretion in immortalized pancreatic β-cells (AG at 0.1 μmol/L) and in cultured mouse islets (AG 1 to 100 pmol/L)[115,128]. It is noteworthy that glucose levels and time of incubation were critical elements mediating AG's effects on insulin release. Accordingly, AG's insulinotropic effects were only detected at glucose concentrations above 8.3 mmol/L[94,115,127,128]. Data obtained in rodents indicate that ghrelin promptly mediates its effects on β-cell function[115]. In the circulation, AG must exert its activity quickly before being degraded. However, *in vitro* AG treatments were carried out for at least 30 min. It is therefore necessary to design experiments allowing the characterization of ghrelin's effects on insulin release in a time-resolved manner. This would allow determining whether ghrelin directly mediates insulin release and/or its synthesis within β-cells.

The effects of AG and UAG on β-cells have been explored to clarify the effects of both ghrelin forms on survival, proliferation and insulin release. It has been demonstrated that both AG and UAG stimulate insulin release in different β-cell lines[129,130]. Furthermore, in response to an intravenous glucose tolerance test, the administration of UAG at 30 nmol/kg was shown to potentiate insulin release in anesthetized rats[131]. Although these effects could not be detected in rat and mouse isolated islets, the inhibitory effect of AG on insulin release was reversed by the combined treatment with UAG[132]. Granata *et al*[130,133] also reported that both ghrelin forms promote cell survival and prevent apoptosis in different β-cell lines. This group also reported that UAG treatment (two subcutaneous administrations of 100 μg/kg for 7 d) could prevent diabetes in newborn rats treated with streptozocin. Although UAG has been shown to influence the release of insulin, important questions remain regarding the mechanisms underlying these effects in the pancreas. For instance, it will be critical to determine whether ghrelin influences the acute release of insulin or its synthesis within β-cells.

The information contained in the above paragraphs suggests that AG inhibits while UAG restores insulin secretion. Although there are many discrepancies in the literature, evidence suggests that the influence of ghrelin on β-cell function depends on the dose of ghrelin used for the treatment as well as the glycemic state under which experiments are carried out. The available data also indicates the relevance of establishing a time-frame during which responses occur. In fact, different groups have described that ghrelin mediates a biphasic response with rapid inhibition and subsequent stimulation of insulin release. Also, homo- and heterodimerization of the GHS-R1a receptor could explain the conflictual observations currently reported in the literature. It is therefore critical to fully determine the (1) optimal doses of AG and UAG; (2) conditions; and (3) the time continuum under which ghrelin influences β-cell functions. Due to its adipogenic nature, it is also of potential interest to investigate whether chronic hyperprolinemia could promote lipotoxicity within β-cells.

EFFECTS OF INSULIN ON CIRCULATING GHRELIN LEVELS

Early after the discovery of ghrelin, an inverse relationship was observed between the ghrelin and insulin levels in animal and human models. In the previous section, the effects of AG and UAG on insulin were reviewed. However, the influence of insulin on both ghrelin forms has also been investigated. It was initially observed that ghrelin levels decrease significantly in healthy participants in response to food intake[134,135]. Moreover, under fasting conditions, ghrelin levels were shown to be inversely correlated with insulin values[79]. Taken together, these elements suggest that insulin could reduce circulating ghrelin levels.

Ghrelin levels have been measured following the intake of different types of meals. However, to isolate the effect of insulin and eliminate potential confounding factors, specific models mimicking postprandial conditions such as the oral glucose tolerance test (OGTT) or the euglycemic hyperinsulinemic clamp (EHC) have been used. It was first reported that total ghrelin levels are significantly reduced in response to OGTT or mixed meals in healthy participants after approximately 35 min[136,137]. In these studies, circulating ghrelin levels were decreased in response to insulin but not following the combined parenteral administration of insulin and glucose[136,137]. These results suggest that decreases in ghrelin levels are not directly mediated by insulin but rather through other mechanisms that require nutrients transiting in the gastrointestinal tract.

Clinical protocols were also designed to study the variations in total ghrelin levels under defined hyperinsulinemic conditions. For instance, in healthy and obese volunteers submitted to EHC or hypoglycemia, total ghrelin levels were significantly reduced[85,138]. Interestingly, in slightly overweight individuals submitted to EHC, total ghrelin concentrations were reduced by 25% and these effects were still detectable 15 min after the insulin infusion ended[139]. Also, under the euglycemic/hyperinsulinemic condition, total ghrelin levels were further reduced by the co-administration with GH and an inhibitor of hormone-sensitive lipase activity in GH-deficient patients[140]. Similar results were observed in response to three-steps hypo-, eu- and hyperglycemic/hyperinsulinemic clamps[141]. Although total ghrelin concentrations were stable before the administration of insulin, the levels of the hormone promptly decreased in response to hyperinsulinemia and remained stable during the hypo- and euglycemic states. However, the most important

reductions in ghrelin levels were noted during the hyperglycemic/hyperinsulinemic conditions. In another study, healthy participants were submitted to three different types of clamps[142]. During the first clamp, hyperglycemia and the resulting elevation of endogenous insulin did not alter ghrelin levels[142,143].

The impact of EHC on ghrelin levels was also studied in different pathological conditions including Pradder-Willi syndrome (PWS), PCOS, and hyper- and hypothyroidism. For instance, elevated total ghrelin levels were reported in children with PWS. The influence of EHC on total ghrelin levels was therefore investigated in both patients with PWS and normal children[144]. Under these conditions, total ghrelin levels were decreased to a greater extent but still remained higher throughout the EHC in patients with PWS compared to controls. Total ghrelin levels were higher in PWS children and their response to EHC was proportional to the one of control individuals. Glucose disposal was similar between normal children and PWS patients, suggesting that under hyperinsulinemic conditions ghrelin levels are reduced in function of the degree of insulin resistance rather than being solely influenced by insulin and glucose levels. To confirm this, patients with type 2 diabetes and healthy individuals were also submitted to EHC. In these patients, fasting total ghrelin levels were lower than in healthy individuals. As expected, total ghrelin levels reduction was significantly less pronounced in patients with type 2 diabetes compared to healthy individuals[145]. This suggests that impairments in IR signaling could disturb the physiological regulation of ghrelin levels. It is recognized that ghrelin levels and insulin sensitivity are lower in women with PCOS. To further study the effect of insulin sensitivity on the regulation of ghrelin levels, women with PCOS were submitted to EHC. Unexpectedly ghrelin levels were not differently modulated in PCOS than in normal women, indicating that the androgen levels could also influence the modulation of ghrelin in this population[146].

Patients with hyperthyroidism also exhibit a negative association between total ghrelin levels and energy expenditure[147]. In these patients, ghrelin levels are also decreased. To investigate the effect of hyperthyroidism normalization, ghrelin levels were measured during EHC before and after medical treatment with antithyroid hormones. Similarly, increased ghrelin levels are observed before and after normalization in patients with hypothyroidism[148]. Despite this difference, ghrelin profiles observed during EHC were not altered by antithyroid treatment or by L-thyroxine (T4) replacement[148,149]. These results indicate that the reduction in ghrelin observed during EHC is independent of thyroid status. The effect of ghrelin on the hypothalamo-pituitary-thyroid axis was also investigated in healthy participants. In contrast to the results obtained in patients who underwent hyper- or hypothyroid normalization, the administration of AG (50 μg) directly increased free T4 while reducing thyroid stimulating hormone concentrations in the circulation[150]. This suggests that the thyroid status does not influence the inhibitory effect of insulin on ghrelin secretion; however ghrelin treatment could directly regulate thyroid functions.

Total ghrelin levels are decreased to a greater extent during EHC in individuals with high insulin sensitivity. However the impact of insulin on the circulating levels of AG and UAG remained uncharacterized for many years. To further characterize the effects of hyperinsulinemia on the different forms of circulating ghrelin, we decided to measure AG and total ghrelin (and estimate UAG levels by subtracting total ghrelin-AG values) during EHC in insulin-sensitive (ISO) and insulin-resistant (IRO) obese postmenopausal women[27]. Total ghrelin and UAG levels were significantly decreased by EHC in ISO and IRO women. However, during EHC, AG levels were significantly reduced only in ISO individuals and the maximal amplitude of reduction was more important than in ISO participants. Similarly, the AG/UAG ratio was significantly lower in ISO women in the fasting condition and throughout EHC. Interestingly, in the total population (ISO + IRO), the maximal amplitude of reduction for total ghrelin and AG were both positively correlated with insulin sensitivity. It was later shown that fasting AG and UAG levels are decreased between the second and the third term of pregnancy in women with diabetes[151]. This was also associated with less important decreases in UAG but not in AG during EHC.

The molecular mechanisms by which insulin regulates ghrelin levels were investigated only in a limited number of studies. Similarly to the results obtained in humans, insulin was shown to reduce total ghrelin levels in rats[152]. Data presented in the signaling section also provided evidence that the gastric insulin signaling activation influences ghrelin mRNA, gastric preproghrelin and circulating ghrelin. Results from two different studies in rodents also indicate that a hyperinsulinemic state could enhance ghrelin mRNA expression but there is no information available on protein levels[31,114]. Although the effects of insulin on total ghrelin levels have been abundantly studied in the literature, it remains that AG and UAG profiles need to be further characterized. Therefore it is critical to decipher the mechanisms mediating the effects of insulin and potential receptor signaling impairments on AG and UAG secretion both in animal and human models under normal and pathological conditions.

CONCLUSION

Although it was discovered more than ten years ago and was the object of an impressive number of publications, important questions still remain regarding the physiological control of AG and UAG secretion and the distinct role of both ghrelin forms in the regulation of metabolic functions. The present work intends to highlight the interrelationships between ghrelin, insulin and glucose homeostasis. Available data indicate that ghrelin influences insulin secretion and vice versa. New evidence suggests the existence of crosstalks between the signaling pathways induced by the activation of the native ghrelin receptor, GHS-R1a and the insulin receptor. However, these interactions seem to oppose themselves

as they are taking place in the central nervous system or in the periphery. This suggests that in different tissues and organs, the heterodimerization of GHS-R1a with Gpr83, DRD1/2, MC3R and potentially other receptors could trigger the activation of distinct signaling pathways. Other important issues were denoted in the literature regarding the insulinotropic effects of ghrelin in cellular, animal and human models. This suggests the critical need to better determine doses under which AG and UAG optimally activate distinct metabolic functions. Taking into consideration the complexity of ghrelin's physiology it is also important to characterize the conditions under which altered responses to AG and UAG are observed. Overall, these clarifications should provide a better understanding of the mechanisms underlying AG and UAG secretion as well as to allow the deciphering of their role in the regulation of distinct metabolic functions.

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P- Reviewers: Kluge M, Owecki M, Vestergaard ET
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Emerging role of protein kinase C in energy homeostasis: A brief overview

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Received: November 26, 2013 Revised: February 18, 2014
Accepted: April 16, 2014
Published online: June 15, 2014

Abstract

Protein kinase C-β (PKCβ), a member of the lipid-activated serine/threonine PKC family, has been implicated in a wide range of important cellular processes. Very recently, the novel role of PKCβ in the regulation of triglyceride homeostasis *via* regulating mitochondrial function has been explored. In this review, I aim to provide an overview of PKCβ regarding regulation by lipids and recently gained knowledge on its role in energy homeostasis. Alterations in adipose PKCβ expression have been shown to be crucial for diet-induced obesity and related metabolic abnormalities. High-fat diet is shown to induce PKCβ expression in white adipose tissue in an isoform- and tissue-specific manner. Genetically manipulated mice devoid of PKCβ are lean with increased oxygen consumption and are resistant to high-fat diet-induced obesity and hepatic steatosis with improved insulin sensitivity. Available data support the model in which PKCβ functions as a "diet-sensitive" metabolic sensor whose induction in adipose tissue by high-fat diet is among the initiating event disrupting mitochondrial homeostasis *via* intersecting with $p66^{Shc}$ signaling to amplify adipose dysfunction and have systemic consequences. Alterations in PKCβ expression and/or function may have important implications in health and disease and warrants a detailed investigation into the downstream target genes and the underlying mechanisms involved. Development of drugs that target the PKCβ pathway and identification of miRs specifically controlling PKCβ expression may lead to novel therapeutic options for treating age-related metabolic disease including fatty liver, obesity and type 2 diabetes.

Key words: High-fat diet; Signal transduction; Obesity; Mitochondrial function; Insulin resistance

Core tip: Nutrition has important long-term consequences for health. It is one of the lifestyle factors that contribute to the development and progression of obesity (increased fat accumulation), diabetes, and cardiovascular diseases. In fact, obesity rates are increasing dramatically worldwide and obesity amplifies the risk of developing various age-related chronic diseases, such as type 2 diabetes and cardiovascular disease. The prevention or management of chronic diseases is a global priority since they constitute a serious strain on health care systems and account for more than half of the deaths worldwide. Although correct lifestyle remains the mainstream solution to this problem, pharmacological strategies are also being actively seeked. Current antiobesity strategies have not controlled increasing epidemic of obesity and obesity-related disorders. We hope that a better knowledge of the molecular players and biochemical mechanism linking dietary fat to fat accumulation and development of glucose intolerance are critically needed. This review examines a way of metabolizing dietary fat into heat instead of storing them as fat, and the possibility that the "browning" of white fat is regulated by a diet-inducible kinase Protein kinase C-β (PKCβ) may help us explore new translational approaches to combat obesity, improve insulin sensitivity and potentially increase longevity. Finally, attenuation of inflammation in fat by PKCβ inhibition could have

profound clinical consequences because of the large size of the fat organ and its central metabolic role.

Original sources: Mehta KD. Emerging role of protein kinase C in energy homeostasis: A brief overview. *World J Diabetes* 2014; 5(3): 385-392 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/385.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.385

INTRODUCTION

Protein kinase C (PKC) family is the largest serine/threonine-specific kinase family known to comprise approximately 2% of the human kinome[1]. PKCs are broadly conserved in eukaryotes, ranging in complexity from a single isoform in budding yeast (Saccharomyces cerevisiae) to 5 isoforms in Drosophila melanogaste and 12 in mammals[2,3]. Three distinct subfamilies can be identified according to their dependency on three combinations of activators: conventional (α, βⅠ, βⅡ, γ) require phosphatidylserine, diacylglycerol, and Ca^{2+}; novel (δ, ε, η, θ) need phosphatidylserine (PS) and DAG but not Ca^{2+}; atypical PKCs (λ/l, ζ) are insensitive to both DAG and Ca^{2+}. PKC isoforms differ in primary structure, tissue distribution, subcellular localization, *in vitro* mode of action, response to extracellular signals, and substrate specificity. The role of individual PKC isoform is thought to be determined through sub isoform-specific activation processes or isoform-specific substrates in the region downstream of the PKC pathway[4]. Specific role of each isoform is beginning to be understood using isoform-specific transgenic and knockout mouse models. PKCs have been extensively discussed in the literature, and the aim of this review is to focus on the functions of PKCβ in the context of obesity and related metabolic syndromes.

REGULATION OF PKCβ ACTIVITY AND EXPRESSION BY LIPIDS

PKCβ is unique among all PKC isoforms in that a single gene locus encodes two proteins, PKCβⅠ and PKCβⅡ, which are generated by alternative splicing of C-terminal exons and are shown to be physiologically relevant[5]. The difference between these two isoforms resides in the C-terminal V5 domains, which still exhibit a moderate homology (45%) at their amino acid sequences[6,7]. PKCβ is highly expressed in the brain and adipose tissue, and widely expressed at a lower level in multiple tissues including liver, kidney, and skeletal muscle. Analysis of the primary structure of PKCβ reveals the presence of four domains conserved across PKC isoforms (C1-C4) and five variable domains that are divergent (V1-V5). Two functional domains have been described: an amino terminal regulatory domain and a carboxyl terminal catalytic domain. The regulatory domain (V1-V3) contains the so-called pseudosubstrate site which is thought to interact

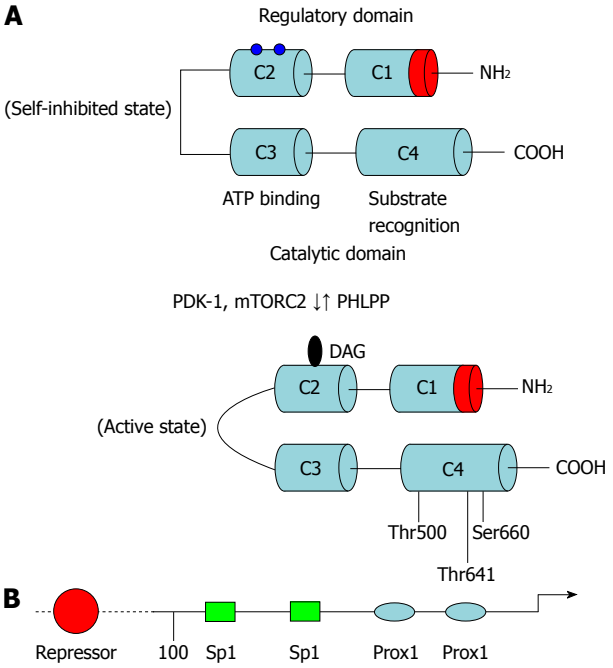

Figure 1 Domain composition of protein kinase C-β and its regulation at the transcriptional and posttranscriptional levels. A: Membrane-targeting modules (C1 and C2), pleckstrin homology domain, the pseudosubstrate region, the kinase core and the C-terminal tail; B: Schematic representation of promoter structure of protein kinase C-β gene. Approximate locations of known regulatory regions are indicated. ATP: Adenosine-5'-triphosphate; PHLPP: PH domain and leucine rich repeat protein phosphatases; PDK-1: 3-phosphoinositide-dependent protein kinase 1.

with the catalytic domain to retain PKCβ in an inactive conformation. The regulatory domain also contains sites for the interaction of PKC with PS, DAG/phorbol ester, and Ca^{2+}. The Ca^{2+} dependency is mediated by the C2 region, while phorbol-ester binding requires the presence of two cysteine-rich zinc finger regions within the C1 domain. The catalytic domain contains two conserved regions, C3 and C4, which are essential for the kinase activity and the binding of adenosine-5'-triphosphate (ATP)/substrate (Figure 1).

In addition to the above specific inputs, other regulatory processes influence the function of PKCβ, including phosphorylation and interaction with specific binding partners. PKCβ is processed by three distinct phosphorylation events before it is competent to respond to the coactivators and is phosphorylated at three conserved serine/threonine residues in the C-terminal domain[8]. Phosphorylation at the activation loop (Thr^{500}) is generally proposed to be first and to be followed by two ordered phosphorylations at the C-terminal tail, the turn motif (Thr^{641} in PKCβⅡ) and then the hydrophobic motif (Ser^{660} in PKCβⅡ). The phosphorylation of the turn motif depends on the mTORC2 complex; this phosphorylation triggers autophosphorylation of the hydrophobic motif[9,10]. The fully-phosphorylated "mature" PKCβ is in a closed conformation in which the pseudosubstrate occupies the substrate-binding cavity, thus autoinhibiting the kinase. Signals that cause hydrolysis of phosphatidylinositol-4,5-bisphosphate result in trans-

location of PKCβ to the membrane by a low-affinity interaction where it binds DAG *via* the C1 domain. Engaging both the C1 and C2 domains on the membrane results in a high-affinity membrane interaction that results in release of the pseudosubstrate, allowing downstream signaling. The membrane-bound conformation is highly phosphatase-sensitive, so that prolonged membrane binding results in dephosphorylation of PKCβ by pleckstrin homology domain Leucine-rich repeat protein phosphatase and PP2A, and subsequent degradation[11]. Binding of Hsp70 to the dephosphorylated turn motif on the C-terminus stabilizes PKCβ, allowing it to become rephosphorylated and reenter the pool of signaling-competent PKC. PKCβ that is not rescued by hsp70 is ubiquitinated by E3 ligases such as the recently discovered RINCK and degraded[12].

PKCβ is also responsive to oxidative stress[13-15]. Why is PKCβ sensitive to oxidative stress? In the PKCβ structure, two pairs of zinc fingers are found within the regulatory domain. They are sites of DAG and phorbol ester binding. Each zinc finger is formed by a structure that is composed of six cysteine residues and two zinc atoms. The high level of cysteine residues renders the regulatory domain susceptible to redox regulation[16,17]. The oxidant destroys the zinc finger conformation, and the autoinhibition is relieved, resulting in a PKCβ form that is catalytically active in the absence of Ca^{2+} or phospholipids[18].

Besides the lipid activation at the post-transcriptional level, PKCβ expression also fluctuates in response to high-fat diet intake. It is shown that feeding high-fat diet (HFD) for 12 wk induces adipose PKCβ expression in an isoform and tissue-specific manner[19]. The molecular mechanism(s) underlying transcription induction have yet to be elucidated but previous studies have cloned and sequenced PKCβ promoter[20-22]. A putative 5'-promoter region for PKCβ is identified and suggested that there is heterogeneity in the active promoter region dependent upon the cellular context. Analysis of the 5'-promoter of PRKCB revealed that a region between -110 bp and -48 bp contains two Sp1 binding sites which are important for basal expression of *PKCβ* gene. In addition two PROX1 sites are also present 3' to Sp1 sites and are involved in inhibiting Sp1-mediated basal transcription of PKCβ promoter[23]. In fact, an inverse relationship between PROX1 and PKCβ levels exist in colon cancer cell lines. It was also found that treatment with a demethylating agent, 5-aza-2'-deoxycytidine, restored PKCβ mRNA expression in PROX1-expressing cells, suggesting that the 5'-promoter of PKCβ is methylated in these cells[23]. Actually, a CpG island in this region, in particular a CpG site within the distal Sp1 site is identified in this study, leading to downregulation of PKCβ transcription. Hypermethylation of PROX1 sites inhibits direct Sp1 binding to this region in PROX1 overexpressing cells. Finally, previous studies have also identified a repressor region located upstream of -110 bp in the PKCβ promoter and the identity of the nuclear factor(s) binding to this region has not been characterized.

NOVEL ROLE OF PKCβ IN LIPID HOMEOSTASIS

A significant conceptual advance in our understanding of the importance of PKCβ signaling in obesity has come from realization that mice deficient in PKCβ express higher levels of genes that regulate fatty acid oxidation and proteins involved in energy dissipation, highlighting its role as a corepressor and in controlling the balance between energy consumption and energy expenditure[24]. On the contrary, genes involved in FA synthesis and gluconeogenesis seem to be downregulated in the absence of PKCβ[25,26]. As a consequence, PKCβ mice are lean, with a significant reduction of body fat and body weight compared to WT mice and are resistant to HFD-induced obesity and hepatic steatosis so that these mice maintain their insulin sensitivity[19]. Moreover, PKCβ levels are shown to be elevated in adipose tissue of leptin-deficient (ob/ob) mice and deletion of PKCβ in ob/ob mice attenuates obesity syndrome of these mice[26]. An important mechanistic insight is the revelation that in PKCβ-deficient mice white adipose tissue (WAT) express genes characteristic of BAT including peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), fatty acid transporter carnitine palmitoyltransferase, and uncoupling protein-1 (UCP-1). Targeted disruption in mice of several genes directly involved in energy metabolism and fat accumulation also leads to lean phenotype with a marked increase in UCP-1 expression in adipocytes, particularly in white fat depots[27-29]. Thus total energy consumption is increased significantly in PKCβ-null mice, presumably as a consequence of energy dissipation in WAT resulting from the expression of UCP-1 and increased mitochondrial activity. The ability of white and brown adipocytes in each depot to reversibly switch into one another has been reported, but the extent to which this occurs and the precise mechanisms involved are not fully understood. The search for regulators that could mediate conversion of white adipocytes (energy storing) into brown adipocytes (energy consuming) has led to the identification of PGC-1α, FOXC2 and positive regulatory domain-containing 16 as transcriptional regulators that have been found to promote a brown fat genetic program, while retinoblastoma protein and RIP140 have been described to favor a white adipose phenotype[27-30]. Another important aspect of these studies relates to possible connection between PKCβ and β-adrenergic receptor levels in WAT. Results presented argue strongly in favor of an inverse relationship between PKCβ and β3-adrenergic receptor expression[26]. The proposed relationship is consistent with earlier reports showing that sustained PKC activation suppressed β-ARs expression at the transcriptional level[31-33]. The net consequence of PKCβ-mediated adipose dysfunction could have profound clinical consequences because of the large size of the fat organ and its central metabolic role. Interestingly, in agreement with the above animal studies, adipose

PKCβ activation is subsequently linked to obese side effects of antipsychotic drugs in humans[34]. Moreover, in agreement with its role in energy homeostasis, PKCβ is shown to be required for adipocyte differentiation[35], PKCβ inhibition promotes insulin signaling in adipocytes[36,37], and PKCβ promoter polymorphism is associated with insulin resistance in humans[38].

The role of PKCβ in obesity is further supported by its potential involvement in angiogenesis. To ensure a sufficient supply of nutrients and oxygen and to transport fatty acids and adipokines, an extended microvasculature is mandatory for adipose tissue. Adipogenesis and angiogenesis are two closely related processes during adipose tissue enlargement, as shown in animal studies and *in vitro* models[39,40]. As adipocyte hypertrophy endures, local adipose tissue hypoxia may occur due to hypoperfusion since the diameter of fat cells overgrows the diffusion limit of oxygen. As a result, hypoxia-inducible transcription factors are expressed triggering the expression of angiogenic factors [vasuclar endothelial growth factor (VEGF), hepatocyte growth factor, plasminogen activator inhibitor-1]. In view of role of PKCβ/HuR in regulating VEGF expression at the post-transcriptional level, simultaneous induction of PKCβ is expected to promote VEGF expression[41,42].

Finally, specific overexpression of a constitutively active PKCβ Ⅱ mutant in mouse skeletal muscle demonstrated that this splice variant of PKCβ not only induces insulin resistance, but also affects the levels of several genes involved in lipid metabolism[43]. Thus impairment in the expression of PGC-1α, acyl CoA oxidase and hormone-sensitive lipase, but enhanced expression of the lipogenic transcription factor sterol response element-binding protein 1c in skeletal muscle, were associated with decreased lipid oxidation and increased intra-myocellular lipid deposition. In addition to these direct effects in muscle, these animals showed defects in insulin action in the liver and brain, as well as hepatic lipid accumulation similar to that seen in fat-fed animals.

POTENTIAL ROLE OF PKCβ IN MITOCHONDRIAL FUNCTION

Several studies have emphasized the association between enhanced mitochondria-derived H_2O_2 and insulin resistance, particularly in the context of excessive nutrient intake that results in metabolic imbalance[44-47]. Oxidative stress has also been described clinically, as well as in WAT of many additional mouse models of obesity, such as the KKAy and db/db mice. Systemic markers of oxidative stress increase with adiposity, consistent with the role of reactive oxygen species (ROS) in the development of obesity-induced insulin resistance. Available data suggest that an increase in ROS significantly affects WAT biology and leads to deregulated expression of inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, and macrophage chemoattractant protein-1, and insulin resistance, which could contribute to obesity-associated diabetes and cardiovascular diseases[47]. Moreover, oxidative stress induced by ROS stimulates fat tissue development both *in vitro* and *in vivo*. H_2O_2-induced oxidative stress is shown to facilitate the differentiation of preadipocytes into adipocytes by accelerating mitotic clonal expansion[48]. Antioxidants such as flavonoids and N-acetylcysteine inhibit both adipogenic transcription factors C/EBP-β and PPAR-γ expression, as well as adipogenic differentiation in 3T3-L1 preadipocytes[49,50]. N-acetyl cysteine (NAC) was also shown to reduce ROS levels and fat accumulation in a concentration-dependent manner[50]. Moreover, animals on a HFD with the antioxidant NAC exhibited lower visceral fat and body weight[51]. Finally, ROS scavenging is associated with fat reduction in obese Zucker rats[52].

Recent studies have highlighted a novel, unexpected signaling pathway bridging the oxidative challenge of a cell to the activation of PKCβ/$p66^{Shc}$-controlled mitochondrial lifespan[53,54]. PKCβ activated by oxidative stress is shown to be required for phosphorylation of the Ser36 of $p66^{Shc}$ and the effect of PKCβ overexpression on mitochondrial Ca^{2+} signaling was not observed in $p66^{Shc-/-}$ cells. Importantly, the mitochondrial consequences of hydrogen peroxide are blocked by hispidine, a specific PKCβ inhibitor. The pathway emerging from these studies is the following: during oxidative stress PKCβ is activated and induces $p66^{Shc}$ phosphorylation, thus allowing $p66^{Shc}$ to be recognized by Pin1, isomerised and imported into mitochondria after dephosphorylation by type 2 protein serine/threonine phosphatase. The $p66^{Shc}$ protein translocated into the appropriate cell domain, can exert the oxidoreductase activity, generating H_2O_2 and inducing the opening of MPTP. This event in turn perturbs mitochondria structure and function. Identification of a novel signaling mechanism, which is operative in the pathophysiological condition of oxidative stress, may open new possibilities for pharmacologically addressing the process of organ deterioration during aging. The above studies are among the first to dissect the downstream target genes and regulatory properties of the PKCβ protein, and therefore make an important contribution to our understanding of the molecular basis to the lean phenotype exhibited by $PKC\beta^{-/-}$ mice. Based on a very recent demonstration that PKCβ/$p66^{Shc}$ mitochondrial axis inhibits autophagy[55] and the evolving role of autophagy in energy homeostasis[56-61], it is possible that a combination of adipose PKCβ activation, mitochondrial dysfunction and insufficient autophagy may contribute to the development of diet-induced obesity. In addition to mitochondrial effects, PKCβ is an upstream regulator of NOX but this signaling axis actively produces superoxide across the membranes of neutrophils and phagosomes[62-65]. Accumulating data so far implicates mitochondria as the main source for regulation of autophagy by ROS production in adipocytes[66], whereas NOX contributes to activation of selective, bacterial autophagy[67] (Figure 2).

Although biological function of PKCβ in energy

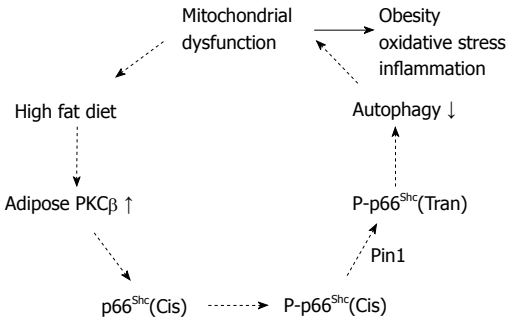

Figure 2 Proapoptotic signals, including reactive oxygen species, activate protein kinase C-β, which in turn phosphorylates p66^{Shc} at serine 36. Phosphorylated p66^{Shc} translocates to the inner mitochondrial membrane and acts as a redox enzyme to amplify oxidative stress by generating H_2O_2. Increased H_2O_2, in turn, causes opening of the mitochondrial permeability transition pore and apoptosis. Protein kinase C-β (PKCβ) activated by reactive oxygen species further induces p66^{Shc} phosphorylation. This event in turn perturbs mitochondria structure and function.

homeostasis appears to be mostly linked with events occurring at the mitochondria, however, increasing evidence has implied a role for this kinase in nuclear functions, suggesting this may be a pathway to communicate signals generated at the plasma membrane to the nucleus. For example, Goss *et al*[68] first showed that PKCβ translocates to the nucleus at G2/M, concomitant with the phosphorylation of lamin B1. Subsequently, a considerable number of nuclear proteins have been identified which are *in vivo* and/or *in vitro* substrates for PKCβ. These proteins include: histone H3, DNA topoisomerase I and Ⅱa, DNA polymerase α and β, cyclic AMP-response element-binding protein, retinoblastoma protein, and vitamin D receptor[69-73]. It has even been shown that PKCβ I co-localizes with androgen receptor and lysine-specific demethylase 1 on target gene promoters and phosphorylation of histone H3 at threonine 6 by PKCβ I is the key event that prevents lysine-specific demethylase 1 from demethylating histone H3 lysine 4[69]. Finally, activated PKCβ indirectly can affect other signaling cascades, including PI3-kinase/Akt pathway, extracellular signal-regulated kinase, and p38 pathway which can impact nuclear events[74-79]. It is thus clear that characterization of PKCβ downstream signaling in the nucleus and its relevance to energy homeostasis is another facets that requires in-depth investigation.

The above findings are applicable to the pathogenesis of obesity and type 2 diabetes since mitochondrial loss in WAT correlates with the development of obesity and type 2 diabetes[80,81]. Indeed, mitochondrial DNA copy number, mitochondrial mass, and mitochondrial activity are all decreased in the white adipose tissue of mouse models of obesity, such as ob/ob and db/db mice[82,83]. Similarly in patients with insulin resistance, type 2 diabetes, and severe obesity, the abundance of mitochondria and the expression of key genes pertinent to mitochondrial function are significantly reduced in white adipose tissue, in concert with decreased adipocyte oxygen consumption rates and ATP production[84,85]. The mitochondrial dysfunction, which could impair substrate oxidation in adipose tissue, is thought to participate in metabolic impairment capacity, thereby accentuating the development of obesity and associated pathologies, such as type 2 diabete. As a result, WAT mitochondria are emerging as highly attractive organelles for therapeutic interventions with the potential to impact upon systemic metabolism. Interestingly, the insulin-sensitizing effects of thiazolidinediones are closely matched by robust increases in adipose tissue mitochondrial biogenesis[86].

CONCLUSION

We have reviewed recent advances pertaining to the potential role of PKCβ in regulating energy homeostasis and contribution to the development of metabolic syndrome. Evidence gathered recently point to an essential role for PKCβ in diet-induced obesity. As a signaling pathway, PKCβ is highly sensitive to changes in environment and fluctuations in lipid supply activate adipose PKCβ, which in turn appears to promote fat accumulation *via* modulating mitochondrial function. A positive loop between oxidative stress and PKCβ/p66^{Shc} is promising and may be the major mechanism underlying contribution of PKCβ activation in generating oxidative stress observed in the obese state. The main gap in our understanding today lies in the specific, molecular and chemical mechanisms of PKCβ-mediated energy homeostasis. What are the mitochondrial and nuclear targets of PKCβ physiologically relevant to energy homeostasis? How is the dietary lipid signals transmitted to the PKCβ promoter? Is PKCβ regulatory signaling network dysregulated in metabolic disease states? Can PKCβ inhibition be adopted to prevent human obesity? These important questions should be the target of future studies. The manipulation of PKCβ levels, activity, or signaling might represent a therapeutic approach to combat obesity and associated metabolic disorders.

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P- Reviewer: Zdravkovic M **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (1): Insulin

Benefits of healthy adipose tissue in the treatment of diabetes

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Received: December 17, 2013 Revised: May 19, 2014
Accepted: May 31, 2014
Published online: August 15, 2014

Abstract

The major malfunction in diabetes mellitus is severe perturbation of glucose homeostasis caused by deficiency of insulin. Insulin deficiency is either absolute due to destruction or failure of pancreatic β cells, or relative due to decreased sensitivity of peripheral tissues to insulin. The primary lesion being related to insulin, treatments for diabetes focus on insulin replacement and/or increasing sensitivity to insulin. These therapies have their own limitations and complications, some of which can be life-threatening. For example, exogenous insulin administration can lead to fatal hypoglycemic episodes; islet/pancreas transplantation requires life-long immunosuppressive therapy; and anti-diabetic drugs have dangerous side effects including edema, heart failure and lactic acidosis. Thus the need remains for better safer long term treatments for diabetes. The ultimate goal in treating diabetes is to re-establish glucose homeostasis, preferably through endogenously generated hormones. Recent studies increasingly show that extra-pancreatic hormones, particularly those arising from adipose tissue, can compensate for insulin, or entirely replace the function of insulin under appropriate circumstances. Adipose tissue is a versatile endocrine organ that secretes a variety of hormones with far-reaching effects on overall metabolism. While unhealthy adipose tissue can exacerbate diabetes through limiting circulation and secreting of pro-inflammatory cytokines, healthy uninflamed adipose tissue secretes beneficial adipokines with hypoglycemic and anti-inflammatory properties, which can complement and/or compensate for the function of insulin. Administration of specific adipokines is known to alleviate both type 1 and 2 diabetes, and leptin mono-therapy is reported to reverse type 1 diabetes independent of insulin. Although specific adipokines may correct diabetes, administration of individual adipokines still carries risks similar to those of insulin monotherapy. Thus a better approach is to achieve glucose homeostasis with endogenously-generated adipokines through transplantation or regeneration of healthy adipose tissue. Our recent studies on mouse models show that type 1 diabetes can be reversed without insulin through subcutaneous transplantation of embryonic brown adipose tissue, which leads to replenishment of recipients' white adipose tissue; increase of a number of beneficial adipokines; and fast and long-lasting euglycemia. Insulin-independent glucose homeostasis is established through a combination of endogenously generated hormones arising from the transplant and/or newly-replenished white adipose tissue. Transplantation of healthy white adipose tissue is reported to alleviate type 2 diabetes in rodent models on several occasions, and increasing the content of endogenous brown adipose tissue is known to combat obesity and type 2 diabetes in both humans and animal models. While the underlying mechanisms are not fully documented, the beneficial effects of healthy adipose tissue in improving metabolism are increasingly reported, and are worthy of attention as a powerful tool in combating metabolic disease.

Key words: Adipose tissue; Diabetes; Insulin-independent; Transplantation; Subcutaneous; Adipokines; Metabolic disease

Core tip: Diabetes mellitus is characterized by perturbation of glucose homeostasis due to insulin deficiency, either absolute or relative. Traditional treatments over the past century have focused on insulin replacement and/or enhancing insulin sensitivity. Ultimate goal in treating diabetes is to re-establish glucose regulation. Recent studies increasingly show the ability of extra-pancreatic hormones, particularly of adipose tissue origin, to compensate for insulin. Adipose tissue is a versatile endocrine organ which, under appropriate circumstances, can exert numerous metabolic benefits and may maintain glucose regulation entirely independent of endocrine pancreas. This review discusses such alternative therapies based on beneficial effects of healthy adipose tissue.

Original sources: Gunawardana SC. Benefits of healthy adipose tissue in the treatment of diabetes. *World J Diabetes* 2014; 5(4): 420-430 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/420.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.420

INTRODUCTION

Diabetes is one of the most serious and widespread metabolic diseases today, affecting 10%-15% of the United States population and 371 million people worldwide. The major characteristics of diabetes mellitus include defects in insulin secretion at the pancreatic β cell level, and defects in insulin sensitivity at the peripheral tissue level. Depending on which of these defects is primary, diabetes is broadly classified into types 1 and 2. Type 1 diabetes (T1D) is associated with absolute deficiency of insulin due to auto-immune mediated destruction of pancreatic β cells, while T2D results in relative or functional insulin deficiency due to gradually progressing resistance to insulin in peripheral tissues. Such resistance leads to initial compensatory hyperinsulinemia and overexertion of β cells, which may progress into absolute insulin deficiency through eventual β cell failure. T1D accounts for 5% of cases, affecting over 2 million Americans and 11-22 million people worldwide, with 78000 new cases diagnosed each year. Characterized by absolute deficiency of insulin resulting in severe hyperglycemia, T1D is fatal if untreated. Available therapies for diabetes, directed at insulin replacement and/or improving insulin sensitivity in peripheral tissues, have various limitations, some of which could be life-threatening. Recent studies demonstrate the ability of healthy adipose tissue to complement or compensate for the function of endocrine pancreas, independent of insulin. Adipose tissue related therapies show promise in overcoming many of the limitations/complications associated with traditional treatments for diabetes.

AVAILABLE THERAPIES

Both type 1 and 2 diabetes are associated with β cell failure due to different mechanisms. Insulin replacement is necessary in all cases of T1D and many cases of T2D. Treatments for T1D primarily focus on insulin replacement, either directly or through transplantation of insulin-secreting tissue such as pancreas or pancreatic islets. Whole pancreas transplantation is currently the most successful means available for achieving long-term insulin independence for T1D patients, and is also helpful in specific cases of T2D associated with significant insulin deficiency[1-3].

Traditional insulin replacement therapies, either direct or through islet/pancreas transplantation, have certain limitations. Direct insulin replacement does not cure the disease and requires repeated administration. A major concern with administration of exogenous insulin is possible overdose, requiring precise monitoring of dosage and blood glucose to avoid fatal hypoglycemic episodes. Whole pancreas transplantation, when successful, provides insulin independence for many years. However it is an invasive surgical procedure not to be undertaken lightly, and carries the risks and complications associated with any major surgery[1,4-7]. Islet transplantation, although a safer and less invasive procedure, is limited by low success rate in the long term due to apoptosis, rejection or poor vascularization of islets. Other concerns include the necessity of large numbers of donor islets and specific complications associated with portal vein cannulation such as portal vein thrombosis and portal hypertension[6-12]. The need for life-long immune-suppressive therapy is also a concern with both islet and pancreas transplantation. Thus, the need remains for better therapies aimed at establishing long-term glucose regulation with fewer complications.

Xenotransplantation of porcine and non-human primate islets has been proposed as a means to overcome the limitations in availability and preservation of human islets. A major challenge with xenotransplantation is hyperactive rejection. Methods proposed to circumvent this problem include encapsulation of islets, and local immunosuppression through genetic manipulation. While long-term graft survival and insulin independence have not yet been achieved, early studies show great potential[13-15]. Recent advances on insulin replacement include generation of insulin-producing cells from embryonic stem cells; transdifferentiation, *i.e.*, generation of endogenous β-cells from non-β-cells using transcription factors that govern pancreatic development; and engineering endogenous surrogate β-cells by tissue-specific insulin gene delivery[15-17]. Stem cell therapy is promising, except for some limitations such as the inability to generate adequate numbers of insulin-producing cells, generation of unnecessary cell types, and harmful side effects such as teratoma formation. In addition to replacing or regenerating insulin-producing cells, another intriguing potential in stem cell therapy is to prevent further destruction of beta cells by appropriately controlling the autoimmune

response. Recent studies describe the potential of stem cell educator therapy for reversal of T1D[18-20]. Human cord blood-derived multipotent stem cells modulate autoimmune responses through altering regulatory T cells and human islet β-cell-specific T cell clones. While suspending the immune response results in significant improvements of glucose regulation, insulin dependence remains an ongoing concern.

Management of T2D includes various agents that improve insulin sensitivity in peripheral tissues, in combination with agents that increase insulin secretion at β cell level. With advancing β cell failure, these treatments have to be combined with insulin replacement or even pancreas transplantation[21-23]. Drugs that improve peripheral insulin resistance include thiazolidendiones and biguanides. While effective in improving insulin sensitivity at varying degrees, these drugs are limited by a number of dangerous side effects including edema, hypertension, heart failure, bone fractures, lactic acidosis and cognitive impairment[21-26]. Complementary strategies include alpha-glucosidase inhibitors which reduce blood glucose by preventing digestion and absorption at gut level. Drugs that increase insulin secretion at β cell level such as sulfonylureas and meglitinides have the same risk of hypoglycemia unawareness as insulin therapy. With progressive β cell failure in T2D the effectiveness of these drugs eventually decreases[23].

A common limitation among all aforementioned approaches is the ongoing need for insulin, and the difficulty of maintaining physiologically appropriate levels and function of insulin after exogenous delivery or endogenous production following different treatments. Studies in the past decade point to the intriguing possibility of insulin-independent glycemic regulation. Although insulin is the major physiological regulator of glucose, numerous extra-pancreatic hormones also exert a powerful influence on glucose homeostasis. Such hormones primarily originate from the gut and adipose tissue[27,28]. While many of these hormones enhance insulin function, some have glucose-lowering actions entirely independent of insulin.

Glucagon-like peptide-1 (GLP-1) is an incretin secreted from entero-endocrine cells in response to food intake. In addition to glucose-dependent augmentation of insulin secretion, GLP-1 has a variety of beneficial effects throughout the body[28-33]. These include insulin-independent effects on glucose metabolism such as direct suppression of glucagon, decrease of hepatic glucose output, decreased absorption *via* delayed gastric emptying and increased glucose uptake by muscle. GLP-1 is also reported to decrease inflammation[29,33,34], decrease cardiovascular risk factors in human patients[35-37], and promote insulin-independent glucose uptake into brown adipose tissue (BAT) in mouse studies[38]. Due to their hypoglycemic effects, analogs of GLP-1 and inhibitors of dipeptidyl peptidase-4 (DPP-4) (enzyme that metabolizes GLP-1) are now widely used as therapeutic agents for T2D[28,29,39-42]. Direct administration of GLP-1 produces acute hypoglycemia and suppression of glucagon in T1D as well[43,44], and GLP-1's anti-inflammatory effects are believed to be potentially therapeutic in correcting insulitis and enhancing beta cell regeneration in T1D[45]. Despite these beneficial effects, incretin therapy also involves risks such as fatal pancreatitis[46,47].

DIABETES AND ADIPOSE TISSUE

Adipose tissue, believed to be merely a storage organ in the past century, is now widely known for its far-reaching metabolic and endocrine functions. Adipose tissue is classified into white and brown fat based on their morphology, embryonic origin and basic function. White adipose tissue (WAT), the large energy reserve distributed all over the body, stores and accumulates fat, whereas BAT localized into a few small depots, metabolizes fat, generates heat and increases overall metabolism. WAT and BAT have distinct embryologic origins and appear at different stages of development. While WAT is believed to originate from mesodermal stem cells, BAT originates from dermatomyotomal precursor cells in common with skeletal muscle, and has an interchangeable developmental relationship with skeletal muscle rather than WAT[48-50]. Due to its function in energy metabolism, BAT is highly vascularized and innervated compared to WAT, giving it the characteristic "brown" appearance. Brown adipocytes contain small multilocular lipid droplets as opposed to the large unilocular droplets found in white adipocytes.

WAT is broadly classified into subcutaneous and visceral fat depots which are then further subdivided according to their specific location[51,52]. Healthy WAT is a versatile endocrine organ that secretes a range of hormones which influence physiological functions at all levels, including nutrient metabolism, satiety signaling, immune/inflammatory response, and angiogenesis[27,52-55]. The major adipokines of importance in metabolic homeostasis are adiponectin and leptin. Adiponectin, well known for its insulin-sensitizing effects on peripheral tissues, is secreted from WAT in micromolar quantities and acts on several receptors such as AdipoR1, AdipoR2, and T-cadherin, enhancing AMP-activated protein kinase and the peroxisome proliferator-activated receptor-α pathway in the liver and skeletal muscle. Adiponectin levels are inversely proportionate to insulin resistance, obesity and diabetes. In addition to insulin sensitization, adiponectin directly increases fatty acid oxidation; inhibits gluconeogenesis; enhances glucose uptake into adipocytes; and exerts anti-inflammatory and anti-atherosclerotic effects, which collectively enhance overall health[27,55-62]. Leptin, long known for its central effects on decreasing appetite and food intake, also increases fat oxidation in many peripheral tissues including liver, adipose tissue and skeletal muscle. Obesity is associated with increased leptin levels and resistance to leptin action, whereas enhanced sensitivity to leptin results in leanness and protection from diet-induced obesity. Non-metabolic effects of leptin include enhancing immune response, pro and anti-inflammatory effects, and angiogenesis[27,53-55,63]. Numer-

ous other hormones of WAT origin, including but not limited to angiopoietin like proteins, apelin, insulin-like growth factor-1 (IGF-1) and visfatin, also have direct or indirect effects on glucose homeostasis through influencing functions such as insulin sensitivity, insulin secretion at beta cell level, glucose uptake in peripheral tissues, lipogenesis/lipolysis, and inflammation[27,52-55,64-68].

Under normal healthy conditions, these extra-pancreatic hormones actively complement endocrine pancreas in overall glucose regulation. However, WAT can exert a beneficial influence only as long as it remains healthy and un-inflamed. Inflammation results in conversion of WAT from a beneficial to harmful organ, which then secrets increasing amounts of hyperglycemic adipokines such as resistin and retinol binding protein 4, and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukins 1 and 6[54,55,69-73]. Such compounds increase inflammation and exacerbate hyperglycemia, leading to a vicious cycle of insulin resistance and T2D. While obesity is generally associated with adipose tissue dysregulation, recent studies show that it is the metabolic dysfunction of adipose tissue which primarily leads to insulin resistance, regardless of the presence of obesity[70]. Such metabolic dysfunction is also associated with decreased sensitivity to leptin and resultant hyperleptinemia. Although leptin generally improves metabolism and leanness, pro-inflammatory properties of leptin would lead to further perturbation of adipose tissue function. One of the primary functions of insulin is lipogenesis and maintenance of adipose tissue. Absence of adequate amounts of insulin results in lipolysis and necrosis of adipocytes. In T1D absolute insulin deficiency results in extensive loss of adipose tissue. Even though T2D tends to be associated with obesity, the adipose tissue in T2D patients is unhealthy, and inflamed with extensive cell death and macrophage infiltration[69-73]. T1D is also characterized by generalized inflammation particularly affecting adipose tissue[74,75]. Thus diabetes is associated with progressive dysfunction of adipose tissue.

Considering the strong correlation between adipose tissue inflammation and metabolic disease, maintaining adipose tissue in a healthy state is critical in preventing metabolic disease, and decreasing inflammation is a promising approach to improve and correct such disorders. A major mechanism of insulin-sensitizing agents such as thiazolidinediones is to reduce inflammation in adipose tissue[76-78]. When human T1D patients are treated with insulin replacement, either directly or through transplantation of insulin secreting tissue, there is recovery of adipose tissue[79,80]. While it is generally believed that insulin is necessary for the maintenance of adipose tissue, our recent research shows that it is feasible to generate and maintain healthy adipose tissue in the absence of insulin, and that healthy adipose tissue can compensate for the function of endocrine pancreas[81-83]. Transplantation of embryonic BAT in the subcutaneous space of diabetic mice results in remarkable regeneration of WAT, decrease of WAT inflammation, and reversal of diabetes.

ADIPOSE TISSUE RELATED THERAPIES FOR T1D

The ultimate cure for T1D is to establish permanent and long-term physiological glucose homeostasis. Considering the limitations associated with insulin replacement, and the remarkable influence of non-pancreatic hormones on glucose regulation, establishing glucose control without insulin is an intriguing and increasingly plausible solution.

Insulin-independent amelioration of T1D includes mono-therapy with specific hypoglycemic adipokines, first reported in the past decade. There is a strong negative correlation between diabetes and plasma adiponectin levels[53-58]. Adiponectin gene expression and plasma levels are increasingly used as predictors of metabolic disease in human patients[84-88]. Administration of adiponectin *via* gene therapy has been long known to improve metabolism in T2D in swine and rodent studies, and a few reports indicate similar results with T1D as well[89-95]. Adiponectin gene therapy with hydrodynamic injection into streptozotocin-diabetic mice resulted in improved glucose homeostasis[90], while long-term central infusion of recombinant adiponectin in normal and pancreatectomized rats resulted in improved metabolic homeostasis through several mechanisms including increase in insulin sensitivity and fat oxidation, and decreases in visceral adiposity, hepatic glucose output and beta cell death[91]. The ability of leptin to correct T1D independent of insulin is now well-documented. As first demonstrated in 2008 by Yu *et al*[96], hyperleptinemia produced by adenoviral transfer results in long-term reversal of T1D in mice. Leptin is now well known to correct T1D independent of insulin in rodent models, primarily through suppression of the hyperglycemic effects of glucagon[96-99]. In both chemically and genetically induced T1D models, leptin administration can produce long-lasting normoglycemia within days of initiation of therapy.

Mono-therapy with other adipokines is also reported to alleviate T1D. Apelin can alleviate complications of T1D in mice, and prevent loss of beta cell mass and alleviate ER stress, major pathogenic mechanisms of T1D[100,101]. In human T1D patients IGF-1 is shown to significantly decrease insulin requirement as well as plasma glucose and HbA1c when used as an adjunct to insulin therapy[102]. Incretin therapy, primarily used in T2D, is shown to have significant benefits in T1D as well. Direct administration of GLP-1 produces acute hypoglycemia and suppression of glucagon in human T1D patients[43,45], and the anti-inflammatory effects of GLP-1 and DPP-4 inhibitors are potentially therapeutic in correcting insulitis and enhancing β cell regeneration in T1D in both rodents and humans[103-106].

While these reports demonstrate the remarkable ability of alternate hormones to complement and/or compensate for insulin, mono-therapy with individual hormones still carries the same complications associated with insulin mono-therapy. Another major barrier in its

applicability to human patients is administration. Gene therapy and adenoviral transfer, as has been used in rodent studies of successful adiponectin and leptin monotherapy, are not viable options due to adverse effects. In addition, adverse effects associated with large supraphysiological doses of these hormones should be kept in mind, including carcinogenesis as has been reported with leptin[107,108]. In addition to the pro-inflammatory and immunogenic properties of leptin, other potential adverse effects include hypertension and thrombosis, and hypoglycemic risk due to excessive suppression of glucagon[63].

Considering the anti-diabetic properties of the aforementioned adipokines when administered alone, it is predictable that a combination of beneficial adipokines at physiological levels would perform better through additive and/or complementary effects, with fewer adverse reactions caused by supraphysiological doses. The feasibility of such an approach is demonstrated in our recent study, where replenishment of healthy WAT following subcutaneous BAT transplants led to reversal of T1D without insulin[81-83]. Transplantation of embryonic BAT into T1D mouse models, chemically or autoimmune induced, results in fast and long-lasting euglycemia accompanied by weight gain, proliferation of subcutaneous WAT, and remarkable decrease of WAT inflammation. These effects are independent of insulin, as indicated by consistently subnormal levels of plasma insulin and drastically low pancreatic insulin content post-mortem. Reversal of diabetes is associated with significant increases of adipokines including adiponectin, leptin and IGF-1, as well as suppression of glucagon. Thus it appears that glucose homeostasis is achieved through a chronic equilibrium of alternate hormones originating from newly replenished healthy WAT[81-83]. Both the severe loss of WAT and inflammation of WAT associated with T1D are corrected by BAT transplants, presumably due to adipogenic and anti-inflammatory factors arising from the transplant. BAT is long known to protect against inflammation as well as improve metabolism[109,110].

Use of BAT transplants to reverse T1D without insulin is a promising step towards simpler and safer therapies for this serious disease. This approach bypasses the serious limitations associated with traditional insulin replacement therapy, such as hypoglycemia unawareness and the need for invasive surgery and/or immunosuppresive therapy. The subcutaneous site is superficial and easily accessible, and can be used for repeated transplants if necessary. Since glycemic regulation is achieved by a physiological combination of endogenously-generated hormones, this approach avoids all limitations in monotherapy with other hormones as well. In addition to the underlying mechanisms being as yet unknown, the major limitation in this technique is the need for embryonic tissue which is currently not applicable in clinical situations. Work in progress include attempts to reproduce the results with adult adipose tissue transplants with appropriate modifications.

ADIPOSE TISSUE RELATED THERAPIES FOR T2D

Metabolic diseases such as insulin resistance, obesity and T2D are characterized by unhealthy adipose tissue, deficient in beneficial adipokines such as adiponectin, and with excess of harmful or inflammatory factors[53-55,69-73]. Recovery from such metabolic disease, through drug therapy, lifestyle changes or surgical intervention, is associated with decrease of inflammation and improved functionality of adipose tissue, including increased secretion of beneficial adipokines[111-118].

Many studies report alleviation of T2D through administration of individual adopokines. Adiponectin gene therapy or hydrodynamic delivery have normalized the metabolic perturbation associated with diet-induced obesity, insulin resistance and T2D in several different animal models including rats, mice and swine[89-95]. In diet-induced diabetic swine, a single injection of purified recombinant human adiponectin resulted in acute decrease of basal blood glucose levels associated with an increase of insulin sensitivity but independent of insulin secretion[89]. Long-term central infusion of recombinant adiponectin in normal rats and pancreatectomized high fat fed rats, a T2D model, resulted in improved metabolic homeostasis through several different mechanisms, including increase in insulin sensitivity and fat oxidation, and decreases in visceral adiposity, hepatic glucose output and beta cell death[91]. Adiponectin gene therapy is also known to ameliorate hypertension associated with obesity in mouse models[92-94]. While there is promise in adiponectin mono-therapy, so far the glycemic regulation has been either transient or not followed for an adequately long period, and administration remains a problem with clinical applications. Mouse studies show that Angiopoietin like proteins improve glucose and lipid homeostasis and alleviate metabolic disease such as T2D, obesity and cardiovascular disease[64,65,119]. IGF-1 administration resulted in remarkable improvement of glucose regulation and insulin sensitivity in human patients with T2D or T1D, even though this therapy is limited by a number of undesirable side effects[102,120]. Leptin is demonstrated to reverse T1D independent of insulin in rodent models[96-99], and recent reports show promising effects on T2D as well[121-123]. However on short term human trials have not yielded positive results so far[121].

As with T1D, transplantation/regeneration of healthy adipose tissue is a potential approach for correction of T2D, insulin resistance and obesity. Several studies on rodent models show improvement of glucose tolerance following transplantation of healthy WAT, in both normal and diabetic subjects[124-130]. Lipoatrophic diabetes, characterized by hyperglycemia and hyperinsulinemia combined with severe loss of adipose tissue, is corrected by transplantation of WAT from healthy donors in a dose-dependent manner[125]. Subcutaneous transplantation of gonadal fat pads from healthy donors into leptin-deficient obese *ob/ob* mice resulted in decrease of obesity,

normalization non-fasting insulin levels and insulin tolerance, and restoration of fertility in females. The results were long-lasting, and dependent on the age and length of leptin deficiency of recipients, and the dose of WAT transplanted[126]. Transplantation of human WAT into leptin-deficient mice resulted in significant improvements in body weight and hepatic steatosis in a dose-dependent manner, associated with increased plasma levels of donor-origin leptin[127]. The importance of the source of WAT is demonstrated in several studies where the removal of visceral fat and replacement with subcutaneous fat, or transplantation of subcutaneous fat from healthy donors, is shown to alleviate or prevent metabolic dysregulation[128-130]. Intra-abdominal and peritoneal transplantation of epididymal WAT prevented the development of age-induced insulin resistance in rats, while transplantation of visceral adipose tissue from normal healthy donors prevented the spontaneous development of T1D and severe fat loss in BB/OK rats in a sex-dependent manner[129,130].

WAT transplantation, while promising, has not yet been successful in complete reversal of metabolic disease. Possible reasons include the inability of WAT transplants to transform inflamed WAT of recipients to a healthy state, as BAT transplants can. In addition there are ongoing problems with transplant rejection and immune response, and maintenance of adipose tissue grafts may be problematic in T1D where adequate insulin is not available to prevent lipolysis. Considering BAT transplants lead to replenishment of WAT without insulin, it is possible that specific factors arising from BAT and/or embryonic tissue may help maintain WAT grafts. Once identified, BAT-derived messengers may prove useful in maintaining WAT transplants. While complete reversal of T1D without insulin has been achieved only with embryonic BAT so far, recent studies show promise in adult BAT transplants in alleviating T2D and obesity. Glucose tolerance in diet induced obese mice is significantly improved through transplantation of inguinal fat pads from healthy donors into the subcutaneous space of recipient mice[131]. High fat diet induced obesity and insulin resistance in mice were reversed by visceral or subcutaneous transplantation of healthy adult BAT, in addition to improvements in glucose tolerance, insulin sensitivity and fat mass[132,133]. Mechanisms include increased glucose uptake into peripheral tissues, increased sympathetic activity and elevated levels of BAT-derived signaling molecules such as FGF21 and interleukin 6.

Another technique to improve the health of adipose tissue is to increase the content of endogenous BAT. There is a well-documented relationship between BAT content and nutritional homeostasis[109,110,134]. Recent studies show that human adults have BAT depots, and that the content of BAT is inversely proportionate to obesity and metabolic disease[135-139]. BAT deficiency in mice results in progressive obesity without hyperphagia, and selective stimulation of β-3 adrenergic receptors, abundantly expressed in BAT, leads to increased energy expenditure and weight loss without affecting food intake[109]. Induction of brown fat lipoatrophy in mice results in increased visceral adiposity associated with excessive secretion of pro-inflammatory cytokines such as TNFα, followed by vascular insulin resistance and vascular dysfunction[139]. Methods such as stimulation of β-3 adrenergic receptors, administration of compounds such as thyroid hormone or atrial natriuretic peptide, and specific BAT-derived messenger molecules, are known to increase endogenous BAT content[140-146]. Thyroxine therapy on a patient with extreme insulin resistance was reported to produce full remission from T2D preceded by proliferation of BAT[140]. Specific transcriptional factors arising from BAT such as PRDM16 are now known to impart BAT-like properties to WAT, *i.e.*, cause "browning" of WAT, which results in overall increase of energy expenditure, decrease of weight gain and improvement of glucose homeostasis as reported in rodent studies[141,142,146]. Another recently identified messenger molecule originating from skeletal muscle, irisin, also improves energy expenditure in mice with no changes in movement or food intake, leading to improvements in obesity and glucose homeostasis[143]. Induction of BAT in WAT depots can also be accomplished with other stimuli, such as cyclo-oxygenase 2 or cardiac natriuretic peptides, leading to increased energy expenditure[144-146]. These studies demonstrate the benefits of increasing endogenous BAT content with various techniques, and overt adverse effects are not yet reported.

CONCLUSION

Taken together, the aforementioned studies demonstrate the powerful global influence of adipose tissue as an endocrine organ, and its strong potential in combating metabolic disease. Adipose tissue is unique in generating a large number of hormones influencing metabolism and inflammation, which may compensate for the function of other endocrine organs upon their malfunction. Recent studies demonstrate the ability of adipose tissue to replace the function of endocrine pancreas under appropriate circumstances. Once the underlying mechanisms are documented such therapies would be applicable to other metabolic disorders as well. Specific characteristics of adipose tissue such as its abundance, accessibility, and extensive ability to regenerate, make it a very useful and convenient source for transplantation.

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P- Reviewer: Kang JH, Saglam F **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

SH2B1 regulation of energy balance, body weight, and glucose metabolism

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Author contributions: Rui L contributed to this work.
Supported by The National Institutes of Health (NIH) grants, No. RO1 DK 065122 and No. RO1 DK091591
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Received: November 25, 2013 Revised: March 6, 2014
Accepted: May 31, 2014
Published online: August 15, 2014

Abstract

The Src homology 2B (SH2B) family members (SH2B1, SH2B2 and SH2B3) are adaptor signaling proteins containing characteristic SH2 and PH domains. SH2B1 (also called SH2-B and PSM) and SH2B2 (also called APS) are able to form homo- or hetero-dimers *via* their N-terminal dimerization domains. Their C-terminal SH2 domains bind to tyrosyl phosphorylated proteins, including Janus kinase 2 (JAK2), TrkA, insulin receptors, insulin-like growth factor-1 receptors, insulin receptor substrate-1 (IRS1), and IRS2. SH2B1 enhances leptin signaling by both stimulating JAK2 activity and assembling a JAK2/IRS1/2 signaling complex. SH2B1 promotes insulin signaling by both enhancing insulin receptor catalytic activity and protecting against dephosphorylation of IRS proteins. Accordingly, genetic deletion of SH2B1 results in severe leptin resistance, insulin resistance, hyperphagia, obesity, and type 2 diabetes in mice. Neuron-specific overexpression of SH2B1β transgenes protects against diet-induced obesity and insulin resistance. SH2B1 in pancreatic β cells promotes β cell expansion and insulin secretion to counteract insulin resistance in obesity. Moreover, numerous SH2B1 mutations are genetically linked to leptin resistance, insulin resistance, obesity, and type 2 diabetes in humans. Unlike SH2B1, SH2B2 and SH2B3 are not required for the maintenance of normal energy and glucose homeostasis. The metabolic function of the SH2B family is conserved from insects to humans.

Key words: Obesity; Type 2 diabetes; Leptin resistance; Insulin resistance; Glucose intolerance; Hypothalamus; Energy balance; Food intake; Hyperphagia; Nonalcoholic fatty liver disease

Core tip: The Src homology 2B (SH2B) family members mediate cell signaling in response to a variety of hormones, cytokines, and growth factors. In the brain, SH2B1 enhances leptin signaling and leptin's anti-obesity action. In peripheral tissues, SH2B1 cell-autonomously enhances insulin signaling. In pancreatic islets, SH2B1 is required for compensatory β cell expansion in response to insulin resistance and β cell stress. SH2B1-deficiency results in severe leptin resistance, energy imbalance, obesity, and type 2 diabetes. SH2B1 mutations are linked to leptin resistance, insulin resistance, obesity, and type 2 diabetes in humans. Thus, SH2B1 is a critical metabolic regulator in mammals.

Original sources: Rui L. SH2B1 regulation of energy balance, body weight, and glucose metabolism. *World J Diabetes* 2014; 5(4): 511-526 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/511.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.511

INTRODUCTION

The Src homology 2B (SH2B) family contains three members (SH2B1, SH2B2 and SH2B3) in mammals. All members contain a characteristic pleckstrin homology (PH) domain and SH2 domain. SH2B1 (also called SH2-B and PSM) was initially identified as a high affinity

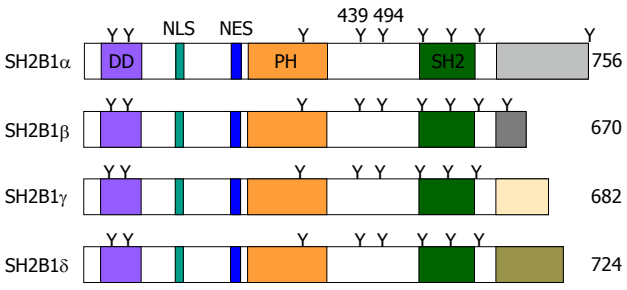

Figure 1 A schematic representation of SH2B1 isoforms. DD: Dimerization domain; PH: PH domain; SH2: SH2 domain; Y: Tyrosine; Numbers: Amino acid numbers.

immunoglobin E receptor (Fc RI) binding protein in the yeast tribrid screen in 1995[1]. SH2B2 (also called APS) was identified as a c-Kit-binding protein by the yeast two-hybrid system in 1997[2]. SH2B3 (also called Lnk) was identified as a SH2 domain-containing, tyrosyl phosphorylated protein in rat lymph node lymphocytes in 1995[3]. The SH2B family is evolutionarily conserved from insects through humans. Unlike mammals, insects have only one *SH2B* gene (also called Lnk)[4,5]. Deletion of SH2B1, but not SH2B2 or SH2B3, results in obesity and metabolic diseases in mice, whereas deletion of either SH2B2 or SH2B3, but not SH2B1, impairs immune function[6-11]. Therefore, individual SH2B1 family members have distinct function in mammals. In this review, I will mainly discuss mammalian SH2B1 and SH2B2.

METABOLIC FUNCTION OF SH2B1

Structure, subcellular localization, posttranslational modification, and tissue distribution of SH2B1

The *SH2B1* gene generates four SH2B1 isoforms (α, β, γ, and δ) through mRNA alternative splicing[1,12-14]. All isoforms have an identical N-terminal region (amino acids 1-632), but differ at their C-termini after the SH2 domain (Figure 1).

SH2B1 structure: All four isoforms have identical dimerization (DD), PH, and SH2 domains (Figure 1). The DD domain mediates SH2B1 homodimerization or its heterodimerization with SH2B2[15-17]. The SH2 domain binds to the phospho-tyrosine motifs of its binding partners (*e.g.*, JAK2 and insulin receptors)[12,18]. The function of the central PH domain remains unclear.

SH2B1 subcellular localization: SH2B1 is located mainly in the cytoplasm, but a subset shuttles between the cytoplasm and the nucleus[19]. SH2B1 contains a nuclear localization sequence (NLS) (KLK^{150}KR) which is required for its nuclear translocation[20]. SH2B1 also contains a nuclear export sequence (NES) (GERWTHRFERL^{231}RLSR) (Figure 1), and replacement of the conserved Leu^{231} and Leu^{233} with Ala increases its nuclear localization[19]. SH2 domain-defective SH2B1β(*R555E*) mutant is also excluded from the nucleus[20]. Therefore, the NLS, NES, and SH2 domain all are involved in the regulation of SH2B1 trafficking between the cytoplasmic and nuclear compartments. SH2B1 is also translocated to the plasma membrane[21]. A N-terminal polybasic region (S^{145}KPKLKKRF), which overlaps the NLS, is required, but not sufficient, for SH2B1β translocation to the plasma membrane[22].

SH2B1 posttranslational modification: SH2B1α and SH2B1β contain nine Tyr residues, and SH2B1γ and SH2B1δ have eight (Figure 1). Tyr^{439} and Tyr^{494} are conserved in all four isoforms, and are able to be phosphorylated by JAK1 and JAK2[23]. Src tyrosine kinases also phosphorylate all four isoforms[24]. Additionally, insulin, insulin-like growth factor (IGF-1), and nerve growth factor (NGF) also stimulate tyrosine phosphorylation of SH2B1 *via* their cognate receptor tyrosine kinases[14,18,25].

SH2B1 contains numerous Ser and Thr residues. NGF stimulates SH2B1 phosphorylation on multiple Ser/Thr residues[21]. Mitogen-activated protein kinase (MAPK) directly phosphorylates Ser^{96}[21], and protein kinase C phosphorylates both Ser^{161} and Ser^{165} residues[22,26]. However, the physiological consequence of SH2B1 phosphorylation remains unknown.

SH2B1 tissue distribution: SH2B1 is ubiquitously expressed in both peripheral tissues and the central nervous system, including adipose tissue, skeletal muscle, liver, pancreas, heart, spleen, hypothalamus, and other brain areas[27]. SH2B1 expression is regulated by neuronal, hormonal, and nutritional signals. The mRNA levels of hypothalamic SH2B1 are 20-fold higher in fed mice than in fasted mice[28]. The expression of hypothalamic SH2B1 in rats is downregulated by high fat diet (HFD) feeding[29]. Chronic overexpression of bovine growth hormone (GH) increases the levels of hepatic SH2B1 protein in GH transgenic mice[30]. The molecular steps, which control the activity of the SH2B1 promoter and the stability of SH2B1 mRNA and protein, remain completely unknown.

SH2B1 regulates cell signaling in response to multiple hormones, growth factors, and cytokines

In cultured cells, SH2B1 acts as an adaptor to couple upstream activators to downstream effectors, to assemble a multiple-protein signaling complex, and/or to enhance the catalytic activity of its bound enzymes.

SH2B1 mediates/modulates leptin signaling: Leptin is an adipose hormone identified by Friedman and his colleagues using positional cloning[31]. Leptin deficiency results in morbid obesity in *ob/ob* mice[31], and recombinant leptin fully corrects obesity and metabolic disorders in *ob/ob* mice[32-34]. Leptin exerts its biological action by binding to and activating its long form receptors (called LEPRb)[35-38]. LEPRb binds to JAK2, a cytoplasmic tyrosine kinase which also mediates GH, prolactin, erythropoietin (EPO), and other cytokine signaling[39,40]. Leptin stimulates tyrosine phosphorylation and activation of JAK2 which activates multiple downstream signaling

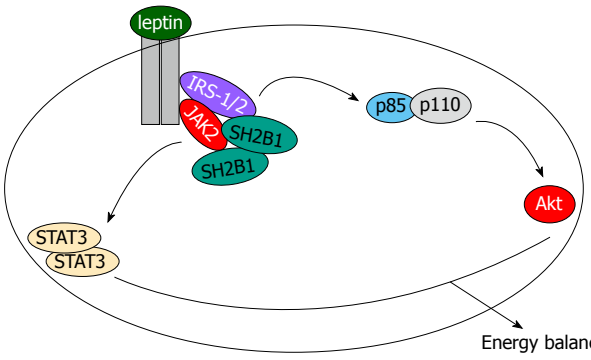

Figure 2 A model of Src homology 2B1 regulation of leptin signaling. The Src homology 2B1 (SH2B1) homodimers bind to JAK2, IRS1, and/or IRS2. SH2B1-JAK2 interaction increases JAK2 kinase activity, thus globally enhancing leptin signaling. JAK2 phosphorylates STAT3 which subsequently homodimerizes, translocates into the nucleus, and activates its target genes. SH2B1-IRS1/2 interaction allows JAK2 to phosphorylate IRS proteins which subsequently activate the PI 3-kinase pathway. Both the STAT3 and the PI 3-kinase pathways are required for leptin to control energy balance and body weight. JAK2: Janus kinase 2; IRS1: Insulin receptor substrate-1; STAT3: Signal transducer and activator of transcription 3.

pathways, including the signal transducer and activator of transcription 3 (STAT3) and the PI 3-kinase pathways[39,40]. Both the STAT3 and the PI 3-kinase pathways are required for leptin's anti-obesity action[39,40]. Impaired leptin signaling and action (leptin resistance) are believed to be the primary risk factor for obesity[39,40].

We reported that leptin stimulates activation of JAK2 which subsequently autophosphorylates on Tyr^{813}[41]. SH2B1 binds *via* its SH2 domain to phospho-Tyr^{813}[41]. This physical interaction markedly increases JAK2 catalytic activity, thus enhancing activation of leptin signaling pathways downstream of JAK2[41-43]. In agreement, leptin-stimulated activation of hypothalamic JAK2 is dramatically attenuated in SH2B1 knockout mice[10]. Leptin sensitivity has been well documented to be negatively regulated by protein tyrosine phosphatase 1B (PTP1B) and SOCS3[39,40]. Overexpression of SH2B1 reverses PTP1B-induced inhibition of leptin stimulation of tyrosine phosphorylation of STAT3[10]. Therefore, cellular leptin sensitivity is likely to be determined, at least in part, by the ability of endogenous SH2B1 to counteract negative regulators such as PTP1B and SOCS3.

Leptin stimulates tyrosine phosphorylation of insulin receptor substrate-1 (IRS1) and IRS2, and IRS proteins subsequently bind to the p85 regulatory submit of PI 3-kinase and activate the PI 3-kinase pathway[39,40,44]. Genetic deletion of IRS2 in LEPR-expressing cells results in leptin resistance and obesity in mice[45]. SH2B1 directly binds to both IRS1 and IRS2 in addition to JAK2[46]. In response to leptin, SH2B1 recruits IRS proteins to JAK2, thus allowing JAK2 to phosphorylate IRS proteins on tyrosine residues[46]. Accordingly, in SH2B1 knockout mice, leptin is unable to stimulate tyrosine phosphorylation of hypothalamic IRS2[10]. SH2B1 is likely to mediate leptin stimulation of the PI 3-kinase pathway by coupling JAK2 to IRS proteins (Figure 2).

SH2B1 C-terminal SH2 domain binds to phospho-Tyr^{813} in JAK2 as discussed above; in contrast, its N-terminal region binds to different sites on JAK2 in a tyrosine phosphorylation-independent manner[43]. Similarly, SH2B1 binds to phospho-tyrosine(s) of IRS1 or IRS2 *via* its SH2 domain, and binds to other sites on IRS proteins *via* its PH domain-containing regions in a tyrosine phosphorylation-independent fashion[46]. SH2B1 forms homodimers or oligomers *via* its N-terminal domains[15-17]. Each individual SH2B1 molecule is able to bind to JAK2 and/or IRS proteins; therefore, SH2B1 dimers or oligomers are predicted to assemble a large signaling complex containing multiple copies of JAK2 and IRS proteins (Figure 2). Physical proximity allows JAK2 to transphosphorylate and activate each other in this complex, contributing to SH2B1 stimulation of JAK2 activation and leptin signaling. Additionally, this highly-organized SH2B1/JAK2/IRS complex may also provide a permissive condition for JAK2 to efficiently phosphorylate IRS proteins and activate the PI 3-kinase pathway in response to leptin.

SH2B1 enhances insulin and IGF-1 signaling: SH2B1 was reported to bind to insulin receptors (IRs) *via* its SH2 domain[18]. Insulin stimulates the binding of SH2B1α to phospho-Tyr^{1158}, Tyr^{1162} and/or Tyr^{1163} within the IR activation loop, and IRs subsequently tyrosyl phosphorylate SH2B1α[13,47]. Overexpression of SH2B1β markedly enhances the ability of insulin to stimulate tyrosine phosphorylation of IRS1 and IRS2[9]. In contrast, SH2B1β(R555E), which has a defective SH2 domain, acts as a dominant negative mutant to inhibit insulin signaling[9]. Moreover, deletion of SH2B1 impairs insulin signaling in the skeletal muscle, adipose tissue, and livers of SH2B1 knockout mice[9].

Mechanistically, SH2B1-IR interaction markedly increases IR catalytic activity and IR-mediated tyrosine phosphorylation of IRS proteins[48]. Replacement of IR Tyr^{1158} with Phe disrupts IR binding to SH2B1, and completely blocks the ability of SH2B1β to stimulate IR kinase activity[48]. SH2B1α similarly increases IR catalytic activity[49]. Additionally, SH2B1β directly binds to tyrosyl phosphorylated IRS1 and IRS2 and protects IRS proteins against dephosphoarylation, thus prolonging the ability of IRS proteins to activate their downstream pathways[48]. Accordingly, overexpression of SH2B1α delays dephosphorylation of IRS proteins in cells[50]. SH2B1 homodimers and oligomers are predicted to simultaneously bind to both IRs and IRS proteins and assemble a large, highly-organized signaling complex, thereby increasing insulin signaling specificity and efficiency.

SH2B1 also binds *via* its SH2 domain to IGF-1 receptors[14], and is predicted to promote IGF-1 signaling in a similar fashion.

SH2B1 enhances TrkA, TrkB and TrkC signaling: Amino acid sequence analysis reveals that like IRs, Trk family members (TrkA, TrkB and TrkC) contain potential SH2B1-binding site(s) within their activation loops. NGF

stimulates both the binding of SH2B1 to NGF receptor TrkA and phosphorylation of SH2B1 on Tyr/Ser/Thr residues in PC12 cells[21,25]. NGF also stimulates the binding of TrkA to both SH2B1 and SH2B2 in primary neurons[51]. SH2B1-TrkA interaction is mediated by the SH2 domain of SH2B1 and phospho-Tyr^{679}, -Tyr^{683} and/or -Tyr^{684} within TrkA activation loop[21,25,51]. Additionally, SH2B1α binds *via* its proline rich regions (amino acids 394-504 between the PH and SH2 domains) to Grb2, contributing to NGF-stimulated activation of the MAPK pathway[51]. Overexpression of SH2B1β also enhances NGF-stimulated activation of Akt in PC12 cells[52].

Brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) similarly stimulates the binding of SH2B1 to TrkB or TrkC, respectively, and they also stimulate tyrosine phosphorylation of SH2B1[51,53,54]. Unlike JAK2 and IRs, TrkB kinase activity is not enhanced by SH2B1[53].

SH2B1 regulates GH, prolactin, and EPO signaling: JAK2 binds to GH receptors and mediates GH signaling[55]. GH stimulates the binding of SH2B1 to JAK2 and robust tyrosine phosphorylation of SH2B1 by JAK2 in 3T3-F442A fibroblasts[12]. GH stimulates JAK2-mediated phosphorylation of SH2B1 on Tyr^{439} and Tyr^{494} residues[56]. Like leptin, GH stimulates phosphorylation of JAK2 on Tyr^{813} which binds to the SH2 domain of SH2B1[57]. SH2 domain-phospho-Tyr^{813} interaction markedly increases JAK2 activity, thus enhancing GH signaling (*e.g.*, phosphorylation and activation of STAT5B)[42,43].

JAK2 also mediates prolactin signaling[58]. Like GH, prolactin stimulates tyrosine phosphorylation of SH2B1[59]. Overexpression of SH2B1β enhances prolactin signaling, including tyrosine phosphorylation of JAK2[59].

Unlike GH, EPO stimulates the binding of SH2B1 to EPO receptors rather than to JAK2[60]. SH2B1 constitutively binds to unphosphorylated EPO receptors under basal conditions, and EPO stimulates phosphorylation of EPO receptors on Tyr^{343} and Tyr^{401} which subsequently bind to the SH2 domain of SH2B1[60]. EPO rapidly stimulates phosphorylation of SH2B1 on Ser/Thr residues[60]. Knockdown of SH2B1 increases EPO-stimulated tyrosine phosphorylation of EPO receptors, JAK2, and ERK1/2, raising the possibility that SH2B1 may negatively regulate EPO signaling[60].

SH2B1 binds to JAK1, JAK2 and JAK3, but it only stimulates JAK2, but not JAK1 and JAK3, kinase activity[61]. Both JAK1 and JAK2 are able to phosphorylate SH2B1 on Tyr^{439} and Tyr^{494}, but Tyr^{439}/Tyr^{494} phosphorylation does not affect the ability of SH2B1 to stimulate JAK2[23]. The JAK family members mediate cell signaling and action in response to numerous hormones and cytokines in addition to GH, prolactin, and EPO, so it is conceivable that SH2B1 may mediate or modulate cellular responses to these hormones and cytokines through interacting with JAK family members.

SH2B1 regulates additional receptor tyrosine kinase signaling: SH2B1 binds *via* its SH2 domain to tyrosyl phosphorylated platelet-derived growth factor (PDGF) receptors in response to PDGF-BB stimulation[62]. PDGF-BB stimulates phosphorylation of SH2B1 on Tyr/Ser/Thr residues[62]. PDGF-BB is able to stimulate tyrosine phosphorylation of all four isoforms of SH2B1[14]. PDGF receptors directly phosphorylate SH2B1 on Tyr^{439} residue[23].

Glial cell line-derived neurotrophic factor (GDNF) stimulates the binding of SH2B1β to GDNF receptor RET through SH2B1β SH2 domain and RET phospho-Tyr^{981} motifs[63,64]. This interaction increases RET kinase activity, RET autophosphorylation, and RET-mediated tyrosine phosphorylation of STAT3[64].

SH2B1 directly interacts with fibroblast growth factor receptor 3 (FGFR3) and is tyrosyl phosphorylated by FGFR3[65]. The SH2 domain of SH2B1 binds to phospho-Tyr^{724} and phospho-Tyr^{760} of FGFR3, and the interaction increases the ability of FGFR3 to phosphorylate and activate STAT5[65].

SH2B1 regulates multiple cellular responses

In cultured cells, SH2B1 has been demonstrated to regulate multiple cellular processes, including migration, proliferation, and differentiation.

SH2B1 regulates actin cytoskeletal reorganization and cell motility: SH2B1 is able to regulate cell morphology, adhesion, and motility through modifying actin cytoskeletal reorganization in cultured cells. SH2B1β is detected in membrane ruffles, filopodia, and focal adhesions[26,59], and is colocalizated with filamentous actin (F-actin) in membrane ruffles[66]. SH2B1β binds *via* both its N-terminal (amino acids 150-200) and C-terminal regions (amino acids 615-670) to F-actin, and promotes actin filament cross-link[59]. SH2B1 directly binds *via* its amino acids 200-260 to the actin-binding protein filamin A[67]. Additionally, SH2B1 binds *via* its amino acids 85-106 to Rac, a critical regulator of actin cytoskeletal reorganization[68].

SH2B1 mediates GH regulation of cell adhesion and migration. GH increases the cycling of SH2B1 into and out of focal adhesions[26], and promotes SH2B1 colocalization with F-actin in membrane ruffles[66]. Overexpression of SH2B1β, but not SH2 domain-defective SH2B1β(R555E), enhances the ability of GH to stimulate both membrane ruffles in 3T3-F442A fibroblasts[59,66] and macrophage migration[56]. In fact, SH2B1β(R555E) blocks GH-induced lamellipodia dynamics in 3T3-F442A cells[68]. Both the N-terminal region (amino acids 85-106) and the SH2 domain of SH2B1β are required for GH stimulation of cell motility[68]. Additionally, SH2B1β mutants lacking Tyr^{439} and Tyr^{494} phosphorylation sites are unable to enhance GH-stimulated membrane ruffling in 3T3-F442A fibroblasts[23] and GH-stimulated motility of RAW264.7 macrophages[56]. SH2B1-Rac interaction is involved in mediating GH-promoted actin cytoskeletal reorganization and cell motility[68].

Overexpression of SH2B1β similarly enhances prolactin-stimulated membrane ruffling[59]. SH2B1 directly binds to filamin A, which appears to mediate prolactin stimulation of membrane ruffling and cell motility[67].

SH2B1 promotes neuronal survival and neuronal differentiation: Overexpression of SH2B1β markedly enhances the ability of NGF to stimulate neurite outgrowth in PC12 cells[25], and both SH2B1α and SH2B2 are able to promote NGF/TrkA-induced neuronal differentiation[51]. In contrast, overexpression of SH2 domain-defective SH2B1(R555E) blocks NGF-induced neuronal differentiation of PC12 cells[25]. SH2B1β mutants lacking either the NES or the NLS also are unable to enhance NGF-induced neuronal differentiation[19,20]. Overexpression of a N-terminal (amino acids 1-499) truncated SH2B1 mutant, which lacks both NES and NLS, induces axon degeneration in NGF-treated primary sympathetic neurons[51]. Moreover, neutralization of endogenous SH2B1 with anti-SH2B1 antibody decreases the survival of primary sympathetic neurons[51]. These observations suggest that the SH2 domain, NES, and NLS all are required for SH2B1 to mediate NGF stimulation of neuronal differentiation and survival.

SH2B1β also enhances GDNF/RET-induced neuronal differentiation of PC12 cells[63,64]. However, the molecular mechanisms, by which SH2B1 promotes neuronal survival, differentiation, and neurite outgrowth, remain largely unknown.

SH2B1 promotes mitogenesis and transformation: All four SH2B1 isoforms are able to increase the mitogenic response to epidermal growth factor, IGF-1, and PDGF-BB[14,69]. SH2B1 increases the ability of RET to promote transformation of NIH 3T3 cells[64]. SH2B1 is abnormally expressed in non-small cell lung cancer (NSCLC) tissues and NSCLC cell lines[70]. SH2B1 overexpression is associated with increased tumor grade, tumor size, lymph node metastasis in NSCLC patients[70].

Neuronal SH2B1 regulates body weight and nutrient metabolism in mice

We reported that genetic deletion of SH2B1 results in severe obesity and type 2 diabetes in mice[9,10].

Central SH2B1 regulates energy balance and body weight: We disrupt the *SH2B1* gene to generate SH2B1 knockout (KO) mice by DNA homologous recombination[9]. Exons 1-6, which encode the N-terminal region of all four isoforms of SH2B1, are replaced by a neo cassette[9]. SH2B1-null mice are hyperphagic and morbidly obese[10]. Both *SH2B1* KO males and females gain more body weights than wild type (WT) littermates after 7 wk of age[10,71]. White adipose tissue mass and fat content are much higher in *SH2B1* KO mice in either C57BL/6 or 129Sv/C57BL mixed congenic background, and the size of individual white adipocytes is also larger in *SH2B1* KO mice[10].

SH2B1 KO mice are extremely hyperphagic, causing obesity[10]. Surprisingly, energy expenditure, as estimated by O_2 consumption and CO_2 production, is also higher in *SH2B1* KO mice than in WT littermates[10]. Accordingly, in the pair-feeding paradigm in which each individually-housed mouse is fed the identical amount of food daily, *SH2B1* KO mice gain less body weights and become leaner than WT littermates[10].

Food intake is controlled largely by the brain, particularly the hypothalamus[72], so we generate SH2B1 transgenic (Tg) mice in which a rat SH2B1β transgene is expressed specifically in neurons under the control of neuron-specific enolase promoter[27]. SH2B1β Tg mice are crossed with *SH2B1* KO mice to generate TgKO mice which lack endogenous SH2B1 in all cell types but express recombinant SH2B1β specifically in neurons[27]. Neuron-specific restoration of SH2B1β into *SH2B1* KO mice fully rescues the hyperphagic and obese phenotypes in TgKO mice[27]. Energy expenditure, which is abnormally higher in *SH2B1* KO mice, is normal in TgKO mice[27]. Furthermore, SH2B1β Tg mice, which contain homozygous SH2B1β transgenes and overexpress recombinant SH2B1β in the brain, resist HFD-induced obesity[27]. These observations indicate that central SH2B1 is a key regulator of energy balance and body weight. Multiple brain areas and neural circuits are involved in the control of energy metabolism and body weight[72]; however, SH2B1 target neural circuits remain unknown.

Surprisingly, Ohtsuka *et al*[11] reported that disruption of SH2B1 did not cause obesity, insulin resistance, and glucose intolerance; however, their subsequent studies show that their *SH2B1* KO mice indeed display insulin resistance and glucose intolerance as we observed in our *SH2B1* KO mice[9,73]. Since *SH2B1* KO mice have high energy expenditure[10], a slight disturbance of food intake is expected to lead to reduction in body weight. Thus, variations in house conditions and other environmental factors may contribute to body weight discrepancy between these studies.

SH2B1 KO mice have relatively normal somatic growth, indicating that SH2B1 is not required for GH stimulation of body growth[9,11,71]. Nonetheless, it is still possible that SH2B1 may modulate GH regulation of metabolism and/or other physiological processes.

Central SH2B1 regulates glucose and lipid metabolism: Obesity is the primary risk factor for insulin resistance and type 2 diabetes[39]. As expected, obese *SH2B1* KO mice develop hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance[9,71]. Insulin signaling is impaired in the skeletal muscle, adipose tissue, and livers of *SH2B1* KO mice[9]. *SH2B1* KO male mice display frank type 2 diabetes after 7 mo of age[9]. Moreover, *SH2B1* haploinsuffiency predisposes to HFD-induced insulin resistance[9]. *SH2B1* KO mice develop the symptoms of metabolic syndrome, including hyperlipidemia, hepatic steatosis, and lipid accumulation in skeletal mus-

cle[10]. Moreover, neuron-specific restoration of SH2B1β reveres obesity, type 2 diabetes, and metabolic syndrome in TgKO mice[27]. These observations indicate that central SH2B1 is absolutely required for the maintenance of normal glucose and lipid homeostasis in mice.

Central insulin and leptin are able to regulate systemic glucose and lipid metabolism independently of their action on energy balance and body weight[39,40,74-77]. SH2B1 positively regulates both leptin and insulin signaling, so central SH2B1 may regulate peripheral glucose and lipid metabolism independently of its action on energy balance and body weight.

Central SH2B1 positively regulates hypothalamic leptin sensitivity: Central SH2B1 controls food intake and body weight at least in part by enhancing leptin sensitivity in the brain. SH2B1 cell-autonomously enhances leptin signaling by promoting JAK2 activity and activation of pathways downstream of JAK2[41]. SH2B1 also mediates leptin-stimulated activation of the PI 3-kinase pathway by binding to IRS1/2 and recruiting IRS proteins to JAK2[46]. *SH2B1* KO mice display severe hyperleptinemia, a hallmark of leptin resistance[10]. Hyperleptinemia develops prior to the onset of obesity, suggesting that leptin resistance is a causal factor for obesity progression in *SH2B1* KO mice[10]. In agreement, exogenous leptin is unable to suppress food intake and weight gain in *SH2B1* KO mice, and has reduced ability to stimulate phosphorylation of hypothalamic JAK2, STAT3 and IRS2 in these mice[10]. Furthermore, neuron-specific expression of recombinant SH2B1β in SH2B1-null mice reverses hyperleptinemia, leptin resistance, hyperphagia, and obesity in TgKO mice[27]. However, neuron-specific expression of SH2B1β(R555E) is unable to rescue leptin resistant, hyperphagic, and obese phenotypes in SH2B1-null mice[78], suggesting that the SH2 domain of SH2B1 is required for its anti-obesity action. Like *SH2B1* KO mice, SH2B1β(R555E) transgenic mice develop obesity, insulin resistance, hyperglycemia, and glucose intolerance[78], suggesting that SH2B1β(R555E) blocks the action of endogenous SH2B1 as a dominant negative mutant.

Orexigenic agouti-related protein (AgRP) neurons and anorexigenic proopiomelanocortin (POMC) neurons in the arcuate nucleus are key leptin targets[39]. Leptin suppresses the expression of AgRP and neuropeptide Y (NPY) but stimulates POMC expression[72]. The expression of hypothalamic AgRP and NPY is higher in *SH2B1* KO mice[10], and neuron-specific expression of SH2B1β in *SH2B1* KO mice normalizes AgRP and NPY expression[27]. By contrast, the expression of hypothalamic POMC is normal in *SH2B1* KO mice[10]. Since *SH2B1* KO mice develop severe hyperleptinemia[10], leptin-stimulated expression of POMC may still be impaired in SH2B1-null mice.

Leptin promotes energy expenditure[39]; therefore, increased energy expenditure in *SH2B1* KO mice cannot be explained by leptin resistance. It is likely that central SH2B1 regulates energy metabolism by an additional leptin-independent mechanism. SH2B1 is able to mediate or modulate cell signaling in response to multiple factors as described above. These pathways may be involved in central regulation of energy balance and body weight. For instance, SH2B1 enhances BDNF signaling[51,54]. Central administration of BDNF suppresses food intake and weight gain; conversely, haploinsufficiency of BDNF or TrkB leads to hyperphagia and obesity in mice[79-83]. Mutations in either BDNF or TrkB are associated with obesity in humans[82,84]. Therefore, neuronal SH2B1 may regulate energy metabolism and body weight by enhancing TrkB signaling in addition to LEPRb signaling in the brain.

Peripheral SH2B1 regulates glucose and lipid metabolism in mice

SH2B1 is expressed in both central and peripheral tissues[27], and peripheral SH2B1 also regulates nutrient metabolism.

Peripheral SH2B1 regulates insulin sensitivity and glucose metabolism: TgKO mice, which lack endogenous SH2B1 in all tissues but express SH2B1β transgenes in the brain, have relatively normal blood glucose, plasma insulin, and glucose tolerance[27]. These observations suggest that peripheral SH2B1 is not required for the maintenance of insulin sensitivity and glucose metabolism in mice fed a normal chow diet. We feed TgKO mice a HFD for 16 wk to induce metabolic stress. TgKO mice develop more severe HFD-induced hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance, even though they have similar body weight and fat content as WT mice[48]. Insulin signaling in skeletal muscle, adipose tissue, and the liver is impaired to a greater extent in HFD-fed TgKO mice[48], and these mice display more severe hepatic steatosis[85]. Thus, peripheral SH2B1 promotes insulin signaling and glucose and lipid metabolism under obesity conditions.

SH2B1 in pancreatic β cells promotes β cell expansion and insulin secretion: Pancreatic β cells express high levels of several SH2B1 isoforms[86]. To examine the role of β cell SH2B1, we generate pancreas-specific *SH2B1* KO (PKO) mice, using the *Pdx1-cre*/loxp system[86]. PKO mice have normal body weight, blood glucose, insulin sensitivity, and glucose tolerance; however, they develop more severe HFD-induced glucose intolerance[86]. Pancreatic insulin content, β cell mass, and glucose-stimulated insulin secretion are significantly lower in PKO than control mice fed a HFD, and PKO islets have more apoptotic cells and less mitotic cells[86]. These observations indicate that SH2B1 in β cells is required for HFD-induced compensatory β cell expansion to counteract insulin resistance in obesity.

SH2B1 appears to directly promote β cell expansion by both promoting proliferation and inhibiting apoptosis[86]. In a rat INS-1 832/13 β cell line, silencing of SH2B1 decreases, whereas overexpression of SH2B1β

increases, β cell toxin streptozotocin (STZ)-induced apoptosis[86]. In line with these findings, PKO mice are more susceptible to STZ-induced β cell destruction, insulin deficiency, and glucose intolerance[86]. Mechanistically, SH2B1 directly enhances insulin and IGF-1 signaling in β cells[86], and both insulin and IGF-1 potently increase β cell survival and proliferation[87-91]. Therefore, β cell SH2B1 cell-autonomously promotes β cell survival, proliferation, and expansion under stress conditions at least in part by enhancing insulin and IGF-1 signaling in β cells.

Hepatic SH2B1 regulates liver triacylglycerol content and very low-density lipoprotein secretion: SH2B1 is also highly expressed in the liver[27], so we generate hepatocyte-specific *SH2B1* KO (HKO) mice using the *albumin-cre*/loxP system[85]. Surprisingly, somatic growth, body weight, insulin sensitivity, and glucose metabolism are similar between HKO and control mice fed either a normal chow diet or a HFD[85]. Adult-onset deletion of SH2B1 in the liver also does not alter insulin sensitivity and glucose metabolism in mice fed a HFD[85]. These data indicate that hepatic SH2B1 is dispensable for the maintenance of systemic insulin sensitivity and glucose metabolism in mice. However, adult-onset deletion of liver SH2B1 decreases liver triacylglycerol content in mice fed a HFD[85], suggesting that hepatic SH2B1 regulates hepatocyte lipid metabolism. Liver-specific deletion of SH2B1 alone does not alter very low-density lipoprotein (VLDL) secretion; however, deletion of liver SH2B1 in SH2B2 knockout mice decreases VLDL secretion[85]. These observations suggest that liver SH2B1 and SH2B2 act redundantly to promote VLDL secretion.

SH2B1 regulates reproduction in mice

SH2B1 is highly expressed in testes and ovaries, and systemic deletion of SH2B1 severely impairs fertility in both male and female mice[11]. Ovary size and follicle number are lower in *SH2B1* KO females; similarly, testis size and sperm number are also lower in *SH2B1* KO males[11]. SH2B1 deficiency impairs both follicle-stimulating hormone and IGF-1 signal transduction in ovaries, which may contribute to impaired fertility in *SH2B1* KO mice[11].

Metabolic function of SH2B1 in humans

SH2B1 rs7498665, the first human *SH2B1* single nucleotide polymorphism (SNP), was reported in 2007[92]. It is associated with hyperleptinemia, increased body weight, increased total fat, and increased waist circumference in a United Kingdom white female cohort[92].

Human SH2B1 is a candidate obesity gene: In 2009, two groups independently reported that *SH2B1* rs7498665 is genetically linked to human obesity in genome-wide association studies (GWAS) on large populations[93,94]. Since then, *SH2B1* rs7498665 has been reported to be associated with human obesity in Swedish adults[95], Belgian adults[96], children of European ancestry[97], Chinese women[98], Hong Kong Chinese[99], Japanese adults[100], the MONIKA/KORA cohort[101], a Mexican cohort[102], and a African-American cohort[103]. *SH2B1* rs7498665 risk allele is associated with increased visceral adiposity in Japanese[104] and German[105]. *SH2B1* rs7498665 is also associated with increased fat intake in Dutch females[106].

Several additional *SH2B1* SNPs have been described since 2009. In GWAS, *SH2B1* rs7359397 is associated with obesity in 249796 adult individuals of European ancestry[107] and in Danish adults[108]. *SH2B1* rs4788102 is associated with obesity in Chinese girls[109] and in Japanese populations[100]. *SH2B1* rs4788099 is associated with increased body mass index (BMI) in individuals of European ancestry[110], and is linked to more servings of dairy products[111]. *SH2B1* rs8055982 is associated with severe obesity in children of European ancestry[97].

Aside from *SH2B1* SNPs, chromosomal 16p11.2 deletion is associated with severe obesity in European cohorts[112-115]. The deleted region contains the *SH2B1* gene. In contrast, chromosomal 16p11.2 duplication is associated with underweight in humans[116].

Several *SH2B1* non-synonymous variants have been identified. *SH2B1* rs7498665 risk allele encodes a non-synonymous substitution of Thr484Ala[92]. However, Thr484Ala substitution alone is not sufficient to cause obesity[117], raising the possibility that other unidentified *SH2B1* mutations, which co-segregate with rs7498665, may increase risk for obesity. Several *SH2B1* missense mutations (P90H, T175N, P322S and F344LfsX20) were reported to be genetically linked to obesity and insulin resistance in mixed European descents[118]. *F344LfsX20A* mutation causes a frameshift, resulting in production of a C-terminally-truncated SH2B1 variant lacking the entire SH2 domain[118]. A separate study reported that *SH2B1* g.9483(C/T) missense mutation, but not Thr175Asp non-synonymous variant (rs147094247), is linked to obesity[119]. *SH2B1* g.9483(C/T) mutation results in generation of non-synonymous SH2B1β(Thr656Ile) and SH2B1γ(Pro674Ser) variants[119]. Four additional rare non-synonymous variants (G131S, V209I, L293R, M465T, and W649G) have been identified in Chinese populations[120]. V209I and M465T variants are detected in obese children, whereas G131S, L293R and W649G variants are observed in lean children[120].

None of the above human SH2B1 variants has been verified in animal models to be a causal factor for obesity or obesity-associated metabolic syndrome. We reported that neuron-specific expression of SH2 domain-defective SH2B1β(R555E), which is functionally similar to F344LfsX20A variant, is sufficient to cause obesity and insulin resistance in mice[78]. These findings raise the possibility that F344LfsX20A non-synonymous variant may be a causal factor for obesity in humans.

***SH2B1* mutations increase risk for type 2 Diabetes in humans:** Obesity is the primary risk factor for insulin resistance and type 2 diabetes[39], so *SH2B1* risk alleles

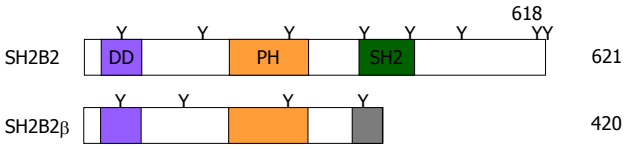

Figure 3 A schematic representation of SH2B2 isoforms. DD: Dimerization domain; PH: PH domain; SH2: SH2 domain; Y: Tyrosine.

are expected to be associated with type 2 diabetes in humans. *SH2B1* rs7498665 is associated with type 2 diabetes in both United Kingdom[92] and French populations[121]. Heterozygous carriers of a P90H, T175N, P322S, or F344LfsX20 non-synonymous variant develop severe early-onset obesity as well as insulin resistance and type 2 diabetes[118].

We reported that peripheral SH2B1 regulates insulin sensitivity and glucose metabolism independently of its action on body weight in mice[48]. SH2B1 in pancreatic β cells directly promotes β cell expansion and insulin secretion in mice[86]. Hepatic SH2B1 regulates liver lipid levels and VLDL secretion[85]. In agreement, *SH2B1* rs4788102 is associated with type 2 diabetes after adjustment for BMI in Japanese[100]. *SH2B1* rs7498665 is associated with increased risk for type 2 diabetes independently of BMI in middle aged Danes[122]. *SH2B1* rs7359397 is associated with insulin resistance after adjustment of BMI in Sweden men at 71 years of age[123]. Thus, SH2B1 also regulates nutrient metabolism by a body weight-independent mechanism.

SH2B1 may regulate multiple physiological processes in humans: Chromosomal 16p11.2 deletion, which results in loss of *SH2B1*, is associated with cognitive deficits, developmental delays[112-115], and autism[115]. Chromosomal 16p11.2 deletion is also linked to abnormal renal and enteric development in humans[124]. *SH2B1* rs4788102 (G/A) is associated with increased circulating triacylglycerol levels in the Northern Swedish Population Health Study cohort[125], and is linked to myocardial infarction[126]. *SH2B1* rs7498665 G allele is linked to increased bone mineral density in Japanese women[127]. However, none of these potential functions have been verified in animal models.

Metabolic function of SH2B1 is evolutionarily conserved

We reported that insulin stimulates the binding of *Drosophila* SH2B (also called Lnk) to Chico, a homologue of mammalian IRS proteins[5]. Almudi *et al*[128] showed that Drosophila SH2B binds to both Chico and insulin receptors in Drosophila cells. SH2B-deficient flies display defects in insulin/IGF signaling, developmental delay, small size, and female sterility[4,5]. Like SH2B1 null mice, SH2B-deficient flies accumulate abnormally-high levels of lipids in their fat bodies[4,5,129].

Interestingly, loss of SH2B increases resistance to oxidative stress as well as lifespan in flies, suggesting that SH2B may regulate aging and longevity[5,129]. However, SH2B1-null mice have a shorter lifespan compared with WT littermates[5]. Obesity and obesity-associated diseases may contribute to early death of SH2B1-null mice. Thus, the role of mammalian SH2B family members in aging remains unclear.

METABOLIC FUNCTION OF SH2B2

SH2B2 was originally identified in 1997[2], and the amino acids of its SH2 and PH domains are 78% and 63% identical to that of SH2B1, respectively.

SH2B2 structure

Crystal structure analysis reveals that the N-terminal region of SH2B2 mediates its homodimerization *via* a Phe zipper[15]. The C-terminal SH2 domain is also able to form a dimer[130]. SH2B2 dimerization is predicted to induce and/or stabilize dimerization of its binding proteins, including JAK2, insulin receptors, or IGF-1 receptors, thus promoting activation of these kinases[15,130].

The *SH2B2* gene also generates an additional C-terminally-truncated isoform (named SH2B2β) through alternative mRNA splicing[131]. SH2B2β contains N-terminal DD and PH domains but lacks C-terminal SH2 domain (Figure 3). SH2B2β binds to both SH2B1 and SH2B2 *via* its DD domain and acts as an endogenous dominant negative variant to inhibit SH2B1 and SH2B2 signaling[131].

SH2B2 regulates insulin signaling and glucose metabolism

Like SH2B1, SH2B2 binds *via* its SH2 domain to phospho-Tyr^{1158} in the activation loop of insulin receptors[130,132,133]. Insulin stimulates phosphorylation of SH2B2 on Tyr^{618} residue in adipocytes[132-134]. Insulin stimulates tyrosine phosphorylation of SH2B2 to a higher level than that of SH2B1[50]. IGF-1 and IGF-Ⅱ also stimulate tyrosine phosphorylation of SH2B2[135]. Additionally, insulin also stimulates Akt-mediated phosphorylation of SH2B2 on Ser^{588} residue[136].

The role of SH2B2 in insulin action is complex. SH2B2 overexpression prolongs insulin-stimulated tyrosine phosphorylation of insulin receptors and IRS proteins[50]. Phospho-Tyr^{618} binds to the tyrosine kinase-binding domain of c-Cbl and promotes c-Cbl phosphorylation by insulin receptors[134,137,138]. Accordingly, knockdown of SH2B2 inhibits insulin-stimulated tyrosine phosphorylation of c-Cbl[139]. SH2B2 also enhances insulin-stimulated phosphorylation of Cbl-b on Tyr^{665} and Tyr^{709} residues[140]. SH2B2 directly binds to SHIP2 and increases SHIP2 activity, and SHIP2 in turn negatively regulates insulin-stimulated tyrosine phosphorylation of SH2B2 and its interaction with c-Cbl[141]. Furthermore, SH2B2 mediates insulin-stimulated plasma membrane translocation of both c-Cbl and Cbl-b in adipocytes[140]. SH2B2 also binds to CAP[134,139] and mediates the activation of the CAP/Cbl/Crk/C3G/TC10 pathway in adipocytes[142]. The SH2B2/CAP/Cbl/Crk/C3G/TC10 pathway is believed to be required for insulin stimulation of GLUT4 trafficking and glucose uptake in adipocytes[142];

consistently, overexpression of SH2B2(Y618F) inhibits insulin-stimulated GLUT4 trafficking[134]. However, SH2B2 also promotes c-Cbl-mediated ubiquitination and internalization of insulin receptors, thus inhibiting insulin signaling[138,143]. Additionally, SH2B2 binds to Asb6, a SOCS family member that may negatively regulate insulin signaling[144].

Deletion of SH2B2 increases insulin sensitivity in mice[6]. We reported that deletion of SH2B2 does not affect HFD-induced insulin resistance and glucose intolerance in *SH2B2* KO mice in either 129Sv/C57BL mixed or C57BL congenic background[71]. Deletion of SH2B2 in *SH2B1* KO mice also does not further exacerbate obesity and insulin resistance in *SH2B1* and *SH2B2* double KO mice relative to *SH2B1* KO mice[71]. The metabolic function of SH2B2 remains unclear.

SH2B2 regulates cytokine signaling and immune response

Like SH2B1, SH2B2 binds *via* its SH2 domain to JAK1, JAK2 and JAK3, and is tyrosyl phosphorylated by these kinases[61,145]. SH2B2 binds *via* both its SH2 domain and non-SH2 domain regions to JAK2, and its SH2 domain binds to phospho-Tyr^{813} of JAK2[16,146]. Unlike SH2B1, SH2B2 is unable to activate, or only slightly activates, JAK2[61,146]. Multiple cytokines, including interferon-γ, EPO, leukemia inhibitor factor, granulocyte-macrophage colony stimulating factor, interleukin-5 (IL-5) and IL-3, stimulate tyrosine phosphorylation of SH2B2, presumably through JAK family members[135,145,147]. Stem cell factor stimulates the binding of SH2B2 *via* its SH2 domain to phospho-Tyr^{568} and -Tyr^{936} of c-Kit and subsequent tyrosine phosphorylation of SH2B2[2,148]. SH2B2 binds *via* its SH2 domain to phospho-Tyr^{343} of EPO receptors[145] , and it also binds *via* its phospho-Tyr^{618} motif to c-Cbl and recruits c-Cbl E3 ligase to EPO receptors, thereby inhibiting the JAK2/STAT5 pathway in hematopoietic cell lines[145]. SH2B2 is colocalized with B cell antigen receptors (BCRs) and negatively regulates BCR signaling, and it is tyrosyl phosphorylated in response to BCR activation[2,149,150].

SH2B1 and SH2B2 play different roles in regulating immune cell function. Deletion of *SH2B1* does not affect the development of T and B lymphocytes and mast cells in mice[11]. In contrast, SH2B2-deficient mast cells display augmented degradulation after cross-linking FcRI[151]. SH2B2 is expressed in B cells but not in T cells[150]. Overexpression of SH2B2 in lymphocytes impairs BCR-induced B cell proliferation and reduces B-1 and B-2 cell number in SH2B2 transgenic mice[150]. Conversely, *SH2B2* KO mice have increased B-1 cell number, and SH2B2-deficient B cells display enhanced response to trinitrophenol-Ficoll, a thymus-independent type 2 antigen[149]. SH2B2 appears to be a negative regulator of a subset of immune cells.

SH2B2 regulates multiple signaling pathways in cultured cells

Like SH2B1, SH2B2 binds *via* its SH2 domain to phospho-Tyr^{679}, -Tyr^{683} and/or -Tyr^{684} of TrkA in response to NGF[51]. BDNF and NT-3 also stimulate the binding of SH2B2 to TrkB and TrkC, respectively[51]. NGF, BDNF and NT-3 stimulate tyrosine phosphoryation of SH2B2[51]. SH2B2 promotes NGF-induced neuronal differentiation of PC12 cells[51].

PDGF-BB stimulates the binding of SH2B2 *via* its SH2 domain to phospho-Tyr^{1021} of PDGFRβ, and SH2B2 in turn inhibits PDGF-stimulated phosphorylation of PLC-γ by competing for phospho-Tyr^{1021} site with PLC-γ[135]. Additionally, PDGF-BB stimulates phosphorylation of SH2B2 on Tyr^{618} which binds to c-Cbl, which recruits c-Cbl E3 ligase to PDGFR complex to negatively regulate PDGFR signaling and PDGFR-promoted mitogenesis[135].

FUTURE DIRECTION

Study of the SH2B family is in its early stages, and many important questions remain unaddressed. Central SH2B1 is required for the maintenance of normal energy balance, body weight, and nutrient metabolism; however, SH2B1 target neurons and neural circuits are unknown. It is unclear whether and how central SH2B1 regulates nutrient mobilization, utilization, and metabolism by a body weight-independent mechanism, and whether and how SH2B1 regulates neuronal activity by a leptin- and insulin-independent mechanism. Numerous *SH2B1* mutations are associated with obesity and type 2 diabetes in humans; however, it is unclear whether these mutations are causal factors for the diseases. Does central SH2B1 regulate higher brain function independently of its action on body weight and metabolism? Do posttranslational modifications affect SH2B1 function? Do SH2B2 and SH2B3 play a role in nutrient metabolism? Can we treat obesity and type 2 diabetes by targeting SH2B family members?

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P- Reviewer: Datta M, Piperi C **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Incretin-based therapies in prediabetes: Current evidence and future perspectives

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Received: August 9, 2014 Revised: September 10, 2014
Accepted: November 7, 2014
Published online: December 15, 2014

Abstract

The prevalence of type 2 diabetes (T2D) is evolving globally at an alarming rate. Prediabetes is an intermediate state of glucose metabolism that exists between normal glucose tolerance (NGT) and the clinical entity of T2D. Relentless β-cell decline and failure is responsible for the progression from NGT to prediabetes and eventually T2D. The huge burden resulting from the complications of T2D created the need of therapeutic strategies in an effort to prevent or delay its development. The beneficial effects of incretin-based therapies, dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists, on β-cell function in patients with T2D, together with their strictly glucose-depended mechanism of action, suggested their possible use in individuals with prediabetes when greater β-cell mass and function are preserved and the possibility of β-cell salvage is higher. The present paper summarizes the main molecular intracellular mechanisms through which GLP-1 exerts its activity on β-cells. It also explores the current evidence of incretin based therapies when administered in a prediabetic state, both in animal models and in humans. Finally it discusses the safety of incretin-based therapies as well as their possible role in order to delay or prevent T2D.

Key words: Type 2 diabetes; Prediabetes; Impaired fasting glucose; Impaired glucose tolerance; Glucagon-like peptide-1; Dipeptidyl peptidase-4 inhibitors; Glucagon-like peptide-1 receptor agonists

Core tip: The beneficial effects of incretin-based therapies on β-cell function in patients with type 2 diabetes (T2D) suggested their possible use in individuals with prediabetes, when greater β-cell mass and function are preserved. Both dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 receptor agonists have demonstrated improvements on β-cell function both in preclinical studies and short-term clinical studies. Until future date for their safety are available, large, long term, prevention trials will be required in order to determine whether they can stabilize or reverse β-cell loss and promote a sustained reduction in the development of T2D in this population.

Original sources: Papaetis GS. Incretin-based therapies in prediabetes: Current evidence and future perspectives. *World J Diabetes* 2014; 5(6): 817-834 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/817.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.817

INTRODUCTION

The prevalence of type 2 diabetes (T2D) is evolving globally at an alarming rate[1]. It is estimated that by the year 2030 approximately 366 million people will have diabetes and more than 90% of them T2D[1,2]. Prediabetes is an intermediate state of glucose metabolism that exists between normal glucose tolerance (NGT) and the clinical entity of T2D[3]. It encompasses both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). IFG is defined by a fasting plasma glucose of 100 mg/dL to 125 mg/dL, while IGT is defined by a 2 h plasma glucose concentration of 140 mg/dL to 199 mg/dL after a 75 g

oral glucose tolerance test (OGTT)[3,4]. Furthermore, the American Diabetes Association suggested that glycated hemoglobulin (A1C) between 5.7% and 6.4% can also be used for the diagnosis of prediabetes, considering that A1C test must be performed by a method that is certified by the National Glycohemoglobin Standardization Program and standardized or traceable to the Diabetes Control and Complications Trial reference assay[4]. Approximately 471 million people worldwide (8% of the world's adult population) are estimated to have IGT by the year 2035[1].

Individuals with IGT have moderate to severe muscle insulin resistance and normal to slightly decreased hepatic insulin sensitivity. They are characterized by defects in both early (0-30 min) and late-phase (60-120 min) of insulin secretion to an oral glucose load[5]. Individuals with IFG have moderate hepatic insulin resistance with normal muscle insulin sensitivity and decreased basal and early phase of insulin secretion[5]. The Veterans Administration Genetic Epidemiology Study and the San Antonio Metabolism (SAM) study have shown a progressive decline in pancreatic β-cell function in individuals with prediabetes[6,7]. The SAM study has demonstrated that when the 2 h plasma glucose during an OGTT was 180-190 mg/dL, β-cell function had already declined by 75% to 80%[6]. Eventually, approximately 20%-34% of the individuals with IFG or IGT progress to T2D over five to six years, while those with combined IFG and IGT have a cumulative incidence of 38%-65%, especially if they have low insulin secretion and severe insulin resistance[8,9]. Relentless β-cell decline and failure is responsible for the progression from NGT to IGT and eventually T2D.

A two to three fold greater increase in plasma insulin response is observed after glucose ingestion compared to a parenteral isoglycemic glucose infusion. This phenomenon was defined as the incretin effect; it accounts for approximately 70%-80% of total insulin release after oral glucose administration[10,11]. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two major incretins described; they account for approximately 90% of the incretin activity[12]. GLP-1 contributes in the overall maintenance of glucose homeostasis through the reduction of glucagon secretion, slowing of gastric emptying and control of body weight, by its appetite suppressant effect[10,11]. GLP-1 levels are significantly decreased in T2D (approximately 50% compared to healthy individuals)[10,13,14]. GIP levels are found to be elevated in patients with T2D as a result of resistance to its biological effects. Sensitivity of β-cells can be resorted after normoglycemia is established, suggesting that resistance to GIP is a manifestation of glucotoxicity[15].

Impairment in incretin hormone secretion/activity in individuals with prediabetes has been reported, although data are not consistent[16-22]. However, reduced GLP-1 levels were reported in the majority of these studies and mainly in subjects with isolated IGT or combined IFG and IGT; early phase GLP-1 response was found to be severely diminished[17-22]. Interestingly, Toft-Nielsen *et al*[22] have shown that during the progression from NGT to IGT and eventually T2D, there is a progressive decline in GLP-1 levels. Early GLP-1 therapy was suggested to preserve β-cell function in subjects with IGT or mild T2D[23].

Native GLP-1 is rapidly inactivated (halfe-life of 1-2 min) by the ubiquitously expressed proteolytic enzyme dipeptidyl peptidase-4 (DPP-4)[10]. The DPP-4 inhibitors are a class of oral antidiabetic agents that improve glycemic control, in patients with T2D, by increasing both GLP-1 and GIP concentrations[24]. GLP-1 receptor (GLP-1R) agonists mimic the actions of GLP-1 and are resistant to DPP-4 degradation; they have achieved significantly lower A1C values in patients with T2D that were associated with significant weight reduction[25]. Studies in cell cultures and animal models demonstrated that both DPP-4 inhibitors and GLP-1R agonists have trophic effects on pancreatic β-cells. Specifically they enhance β-cell proliferation, regeneration and differentiation; thus they increase β-cell mass. They also inhibit β-cell apoptosis, including human β-cells, through inhibition of the caspase pathway[24-26]. The identification of their antiapoptotic properties, combined with observations of β-cell function preservation and sustained glycemic control during their administration, suggested their possible use as early in the clinical course of T2D as possible or even earlier in order to prevent the onset of this disease[27]. The present paper summarizes the main molecular intracellular mechanisms through which GLP-1 exerts its activity on β-cells. It also explores the current evidence of incretin-based therapies, DPP-4 inhibitors and GLP-1R agonists, when administered in a prediabetic state both in animal models and in humans. Finally it discusses the safety of incretin-based therapies, as well as their possible role in order to delay or prevent T2D.

MAIN MOLECULAR INTRACELLULAR MECHANISMS OF GLP-1 ACTIVITY ON THE PANCREATIC β-CELL

Increased glucose levels are first transported into the β-cell by the type 2 facillitative glucose transporter (GLUT-2) and are phosphorylated by glucokinase to glucose-6-phosphate, promoting an increased rate of aerobic glycolysis; this in turn generates substrates (mainly pyruvate) for mitochondrial oxidative metabolism. Glycolytic and mitochondrial respiration promotes an increased cytosolic adenosine triphosphate (ATP)/adenosine diphosphate (ADP) concentration[28]. This major cellular metabolic signal provides the link between glucose stimulus and insulin secretion. The increase of ATP/ADP ratio promotes the closure of ATP-sensitive K^+ channels (K_{ATP}), thereby initiating plasma membrane depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs), Ca^{2+} influx and an increase in the intracellular Ca^{2+} concentration. This in turn stimulates the granules that contain insulin and promotes their release into the

blood compartment. Repolarization of β-cells is mainly mediated by Ca^{2+}-sensitive voltage-depended K^+ (K_{Ca}) channels and voltage-dependent K^+ (Kv) channels. These channels open after glucose-induced membrane depolarization so as to restore the outward flux of K^+[29].

GLP-1 is a 30-amino acid peptide produced in the intestinal epithelial L-cells of the distal ileum and colon by differential processing of the proglucagon gene from the prohormone convertase PC1/3[30]. GLP-1 binds to GLP-1R, a class 2 G protein-coupled receptor, in the cell membrane of the pancreatic islets[31]. Through this receptor it mainly exerts its insulinotropic activity, which is strictly glucose-depended. Specifically, it stimulates adenylate cyclase resulting in the production of cyclic adenosine 3',5'-monophosphate (cAMP). Downstream effectors of cAMP include protein kinase A and the cAMP-regulated guanine nucleotide exchange factor Ⅱ. Through the activation of these two important cellular pathways GLP-1 enhances and amplifies insulin secretion *via* its effects on ATP/ADP concentration ratio, K_{ATP} channels, Kv and K_{Ca} channels, VDCCs, Ca^{2+} influx and intracellular concentrations and insulin granule exocytosis or priming[32,33]. In this way GLP-1 restores glucose-depended insulin secretion in metabolically compromised β-cells; it promotes the induction of glucose competence (Figure 1)[34,35].

In addition to its insulinotropic effects, GLP-1 acts as β-cell growth factor. After binding to its receptor, GLP-1 induces the transactivation of the epidermal growth factor receptor, which activates phosphatidylinositol-3 kinase (PI3-K) and its downstream targets protein kinase B (PKB/Akt), extracellular signal-related kinase, p38 mitogen-activated protein kinase (MAPK) and protein kinase Cζ [36,37]. Through these pathways GLP-1 exerts its action on β-cell proliferation and survival. Moreover GLP-1 promotes an increased expression and activity of the pancreatic and duodenal homeobox-1 (*PDX-1*) gene; hence it increases total PDX-1 levels and promotes its translocation to the nucleus[38]. PDX-1 is of major significance for most of the proliferative, glucoregulatory and cytoprotective actions of GLP-1. It regulates the expression of genes important for β-cell function such as insulin, GLUT-2 and glucokinase. It also replenish β-cell insulin stores and in a long term basis it prevents β-cell exhaustion[38-42]. Moreover, GLP-1 stimulates β-cell proliferation through CREB-mediated *Irs2* gene expression, leading to activation of PI3-K/PKB signaling pathway[43]. Its proliferative activity was also related to insulin growth factor (IGF)-1 expression and autocrine IGF-2 secretion by the β-cell[44]. Furthermore, GLP-1 prevents β-cell apoptosis, induced by a variety of cytotoxic stimuli, and enhances β-cell survival[26,45,46].

DPP-4 INHIBITORS IN A PREDIABETIC STATE

Vildagliptin

Studies organized in animal models: Vildagliptin (LAF237) is an oral agent that inhibits DPP-4 and increases both active GLP-1 and GIP levels; it achieved improved glycemic control in patients with T2D[47]. Five-week-old female C57BL/6J mice were fed with a high-fat diet, as a model of IGT and T2D, or a normal diet for 8 wk[48]. After 4 wk, the mice were treated with vildagliptin in their drinking water (approximately 3 μmol per day per mouse). Controls were given only water. All mice were subjected to an OGTT after 4 wk of treatment. In both high-fat diet-fed mice and the normal diet-fed mice, administration of vildagliptin improved glucose tolerance in association with markedly augmented insulin secretion.

Vildagliptin was also administered in anesthetized obese insulin resistant cynomolgus monkeys in a dose of 1 μmol/kg[49]. Each animal received two OGTTs 45 min after oral administration of vildagliptin or vehicle, 3 wk apart. Plasma DPP-4 activity was inhibited by 82% with vildagliptin therapy ($P < 0.001$) and remained suppressed throughout the duration of the OGTT. Peak plasma GLP-1 levels in the vildagliptin group were significantly higher than those in the vehicle-treated animals, after the glucose load was given ($P < 0.001$). Vildagliptin reduced glucose excursions during OGTTs compared to the vehicle ($P < 0.05$). There was also a trend towards an enhanced insulinogenic response to glucose after vildagliptin therapy.

Clinical studies: Although incretins are stimulated during an oral challenge, it was postulated that due to the long half-life of DPP-4 inhibitors, basal levels of active GIP and GLP-1 could play a role in the improvement of β-cell function in individuals with IFG. Vildagliptin was investigated in a single-blind, single-treatment design study, in which 22 individuals with IFG were enrolled. The drug was administered in a dose of 100 mg daily for 6 wk. Two weeks of placebo treatment before (running period) and after (washout period) the 6 wk were also studied[50]. Treatment with vildagliptin resulted in a slight increase in fasting GIP but not GLP-1 levels, while marked increases of both intact GLP-1 and GIP levels during a meal tolerance test were reported. Fasting plasma glucose (FPG) levels were not significantly reduced. Incremental area under the curve (AUC) of glucose and 2 h glucose decreased after a meal tolerance test. Although AUC of C-peptide and insulin responses did not change significantly, when the decrease in glucose levels was taken into consideration, both markers were improved. Since a formal OGTT was not performed in the population enrolled, the possibility that some individuals had combined IFG and IGT could not be excluded. The disposition index (DI) was increased by 69% and insulin sensitivity by 25% after an intravenous glucose tolerance test (IVGTT), suggesting an improvement of β-cell function when no dynamic change in incretin release would be expected to occur. However, after the 2-wk washout period, all the beneficial effects observed returned to baseline levels.

In a multicenter 12-wk double-blind study 179 individuals with IGT were randomized to receive either

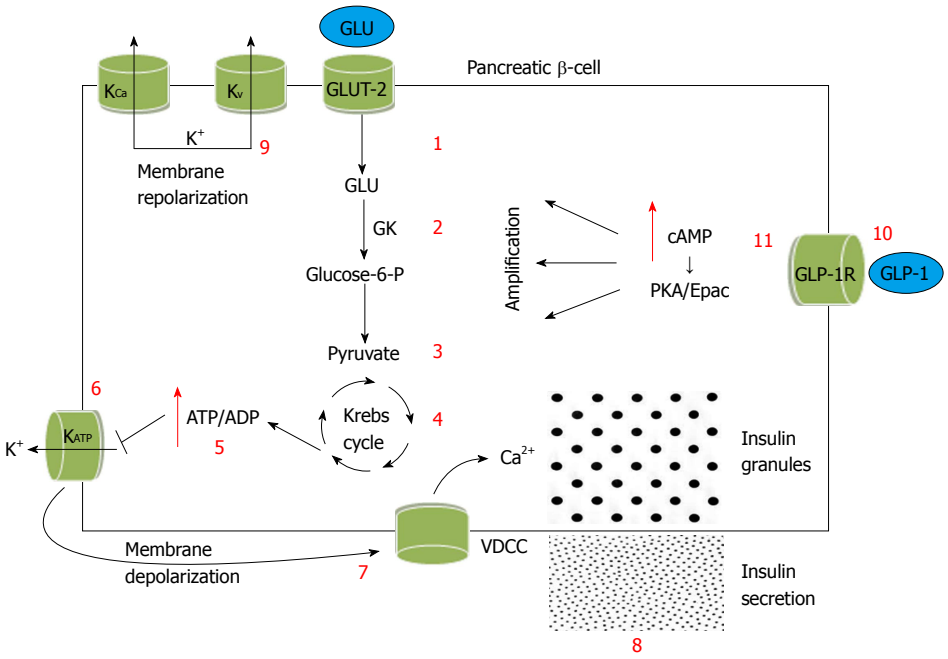

Figure 1 Glucagon-like peptide-1 and the β-cell: Amplification of the glucose-stimulated insulin secretion. Increased glucose levels are transported into the β-cell by GLUT-2. They are phosphorylated by GK to glucose-6-P, promoting an increased rate of aerobic glycolysis. Pyruvate is the main substrate for mitochondrial oxidative metabolism. Increased cytosolic ATP/ADP concentration is the major cellular metabolic signal between the glucose stimulus and insulin secretion. It promotes the closure of K_{ATP} channels, thereby initiating plasma membrane depolarization, activation of VDCCs, Ca^{2+} influx and an increase in the intracellular Ca^{2+} concentration. This in turn stimulates the granules that contain insulin and promotes their release into the blood compartment. Repolarization of β-cells is mainly mediated by K_{Ca} and K_v channels. GLP-1 binds to GLP-1R, a class 2 G protein-coupled receptor, in the cell membrane of the pancreatic cells. Through this receptor it mainly exerts its insulinotropic activity. It promotes increased levels of cAMP through stimulation of adenylate cyclase. Downstream effectors of cAMP are PKA and Epac. Through the activation of these two important cellular pathways GLP-1 amplifies insulin secretion *via* its effects on ATP/ADP concentration ratio, K_{ATP} channels, K_v and K_{Ca} channels, VDCCs, Ca^{2+} influx and insulin granule exocytosis. GLU: Glucose; GLUT-2: Type 2 facillitative glucose transporter; GK: Glucokinase; Glucose-6-P: Glucose-6-phosphate; K_{ATP}: ATP-sensitive K^+ channels; VDCCs: Voltage-dependent Ca^{2+} channels; K_{Ca}: Ca^{2+}-sensitive voltage-depended K^+ channels; K_v: Voltage-dependent K^+ channels; GLP-1: Glucagon-like peptide-1; GLP-1R: Glucagon-like peptide-1 receptor; cAMP: Cyclic adenosine 3',5'-monophosphate; PKA: Protein kinase A; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate.

vildagliptin 50 mg/daily (n = 90) or placebo (n = 89)[51]. Approximately 80% of the patients were IFG and IGT. In individuals receiving vildagliptin there was a marked and sustained increase in active GLP-1 and GIP levels compared to the placebo group (5-fold and almost 2-fold increases in the incremental AUCs for GLP-1 and GIP, respectively). These effects were associated with significant improvements in β-cell function, as estimated by insulin secretion relative to that of glucose (insulin secretory rate AUC0-2 h/glucose AUC0-2 h, mean change between groups 6.1 ± 2.0 pmol/min per meter per millimoles per liter, P = 0.002). Improvements were also reported in α-cell function [glucagon ΔAUC0-2 h, mean change between groups (-3.0 ± 2.0 pmol/L per hour, P = 0.003)]. These beneficial effects contributed approximately to 30% reduction of ΔAUC for glucose. Vildagliptin was well tolerated with a good safety profile and no hypoglycemia was documented.

A three month, double-blind, placebo-controlled study was organized in a population of 48 stable renal transplant recipients, at least six months after transplantation, with newly diagnosed IGT[52]. Participants were randomized to receive 50 mg of vildagliptin, 30 mg of pioglitazone or placebo in a 1:1:1 ratio (16 individuals in each group). There was not any significant difference in corticosteroid therapy between the three groups. Baseline A1C was lowest in the vildagliptin group and higher in the pioglitazone group (P = 0.01). A1C reduction was statistically significant between treatment groups and placebo (placebo *vs* pioglitazone: -0.17% ± 0.33% *vs* +0.09% ± 0.26%; P = 0.013; placebo *vs* vildagliptin: -0.11% ± 0.25% *vs* +0.09% ± 0.26%; P = 0.049). Vildagliptin and pioglitazone reduced the 2 h plasma glucose at three months compared with baseline (vildagliptin: -20 ± 24 mg/dL; P = 0.002 and pioglitazone: -23 ± 29 mg/dL; P = 0.004), while only pioglitazone slightly reduced FPG.

Sitagliptin

Studies organized in animal models: Sitagliptin is the first DPP-4 inhibitor introduced in clinical practice[53]. Sitagliptin and glyburide were administered in obese prediabetic spontaneously hypertensive rat-obese (SHROB) in order to investigate whether it could reverse the metabolic abnormalities in the secretion of both insulin and glucagon[54]. Sitagliptin was found to normalize glucose tolerance following an OGTT, at least as effective as glyburide, in this rat model of metabolic syndrome and prediabetes. Sitagliptin also restored the first phase of insulin secretion after an OGTT more effectively than glyburide. Fasting glucagon levels, which were elevated

in the SHROB model, were normalized after 5 wk of sitagliptin therapy. Fasting insulin and liver glucogen levels were not affected by both drugs. It was suggested that if sitagliptin actions could extend to human prediabetics, then sitagliptin might delay the onset of diabetes[54].

Sitagliptin was also administered in a mouse model of diet-induced obesity with increased FPG and postprandial hyperinsulinemia[55]. It was reported that 12-wk of sitagliptin therapy improved glucose tolerance, reduced FPG, and lowered plasma insulin in randomly fed mice compared with untreated insulin-resistant obese mice. A significant reduction in glucose excursions during an intraperitoneal glucose tolerance test was found. Sitagliptin was also shown to induce a change in the islet size distribution. Specifically, a significantly higher percentage of small islets and a reduced relative percentage of very large islets (due to the very high-fat diet) was demonstrated. This result may explain the better insulin secretory response observed after sitagliptin therapy in response to an *in vitro* glucose challenge.

An animal model with clinical and metabolic characteristics similar to those of individuals with IGT was recently studied[56]. Fructose administration to normal rats for 21 d induced insulin resistance, IGT, hypertriglyceridemia and decreased β-cell mass, due to an increased percentage of apoptosis. The control group was consistent of rats that were fed with a standard commercial diet. Homeostasis model assessment for insulin resistance (HOMA-IR) and for β-cell function (HOMA-β) decreased to almost control values after sitagliptin therapy. Sitagliptin significantly increased β-cell mass by 68%, attaining values close to those measured in standard commercial diet fed rats; inhibition of β-cell apoptosis was the main cellular mechanism for this effect. These changes were associated with normalization of IGT and liver triacylglycerol content.

Clinical studies: In a double blind placebo-controlled trial 22 individuals with IFG, after a baseline meal study, received sitagliptin 100 mg daily (n = 11) or placebo (n = 11) over an 8-wk treatment period[57]. They underwent a second meal study at the end of the treatment period. Sitagliptin did not alter fasting but increased postprandial intact GLP-1 concentrations, while total postprandial GLP-1 concentrations were reduced. Both fasting and postprandial glucose values were unchanged with sitagliptin therapy. Although sitagliptin resulted in a slight improvement in β-cell function (a slightly increased DI was found), this was not sufficient to alter glucose uptake and production and overcome the defect on insulin action. It was speculated that the limited ability of DPP-4 inhibitors to increase insulin secretion in IFG could be due to their glucose depended mechanism, since glucose concentrations are only modestly elevated in IFG. This speculation can also explain the differing effectiveness of sitagliptin on postprandial concentrations in this study compared to other studies in individuals with IGT, with higher postprandial glucose concentrations.

A four week open-label, parallel group study investigated the effects of sitagliptin on insulin secretion and endogenous glucose production in individuals with IFG and no history of prior antidiabetic therapy[58]. Twenty-three individuals with either IFG (n = 10) or NGT (n = 13) were studied by a fasting glucose test and OGTT. All participants received open-label sitagliptin 100mg once daily for 4 wk. Treatment with sitagliptin resulted in a small but significant decrease in FPG compared to baseline in both groups ($P < 0.05$). Endogenous glucose production was unchanged after 4 wk of sitagliptin therapy. Administration of sitagliptin did not altered insulin or glucose excursions in the post-intervention OGTT, but did increase AUC for active GLP-1 and C-peptide compared to baseline levels ($P < 0.01$ for both). Insulin sensitivity and β-cell response indices remained unchanged after administration of sitagliptin.

Beta-cell function in Glucose abnormalities and Acute Myocardial Infarction was a 12-wk multicentre, double-blind, randomized, parallel group study that investigated the effects of sitagliptin 100 mg daily (n = 34) compared to placebo (n = 37) in 71 patients with acute coronary syndrome having IGT or T2D[59]. Investigation of β-cell function was achieved using the insulinogenic index (IGI) derived from an OGTT and acute insulin response to glucose (AIRg) after a frequently sampled IVGTT. At the time of randomization 71% and 62% of the individuals in the sitagliptin and the placebo group had IGT, while 29% and 38% had T2D, respectively. IGI increased significantly, from baseline to 12 wk (9.9 pmol/mmol to 85.0 pmol/mmol) in the sitagliptin group compared to the placebo group (66.4 pmol $mmol^{-1}$ to 58.1 pmol/mmol, P = 0.013). The AIRg increased significantly in the sitagliptin group compared to the placebo group: 1909 pmol L^{-1} per minute *vs* 1043 pmol/L per minute ($P < 0.0001$). During the OGTT and the frequently sampled IVGTT, glucose levels were significantly lower in the sitagliptin arm compared to the placebo arm. Immediate insulin response was higher after sitagliptin therapy, while it remained unchanged after placebo. By 12 wk, 76%, 18% and 6% of the participants in the sitagliptin group had NGT, IGT and T2D respectively. In the placebo arm 41%, 35% and 24% of the participants had NGT, IGT and T2D respectively.

Other DPP-4 inhibitors

Alogliptin is the newest DPP-4 inhibitor approved for T2D therapy, either alone or in combination with other antidiabetic agents[60]. It was administered alone or in combination with voglibose in prediabetic *db/db* mice[61]. Specifically, 6 wk old prediabetic db/db mice were fed with a powder CE-2 diet containing 0.001% voglibose alone (equivalent to 1.8 mg/kg per day), 0.03% aloglitpin alone (equivalent to 72.8 mg/kg per day), or combination of both agents (equivalent to alogliptin: 53.8 mg/kg per day + voglibose: 1.8 mg/kg per day) for 27 d. Control *db/db* and non-diabetic *db/+* mice were fed by a drug-free powder CE-2 diet (vehicle). Plasma DPP-4 activity

was reduced significantly by 18%, 72% and 80% and plasma active GLP-1 levels were increased significantly by 1.8, 4.5 and 9.1-fold in voglibose, alogliptin and combination treated *db/db* mice, compared with vehicle treated *db/db* mice, respectively. Pancreatic insulin content was increased significantly by 3.4, 1.8 and 8.5-fold and A1C was reduced significantly by 1.6%, 0.5% and 2.1% in voglibose, alogliptin and combination treated *db/db* mice, compared with vehicle treated *db/db* mice, respectively. Although quantitative analysis was not preformed, combination treatment resulted in an increased pancreatic insulin staining, PDX-1 staining and GLUT2 membrane localization in β-cells. It also maintained normal distribution of β/α-cells in islets; it was suggested that this combination could preserve pancreatic β-cells in *db/db* mice[61]. The combination of alogliptin and pioglitazone was also found to improve glycemic control and increase pancreatic insulin content in *ob/ob* mice; however the addition of alogliptin to pioglitazone therapy did not contributed to the prevention or the delay of T2D onset in UCD-T2DM rats[62,63].

The effects of chronic administration of the DPP-4 inhibitor FE 999011 were investigated in both obese and insulin resistant fatty Zucker rats and Zucker diabetic fatty (ZDF) rats[64]. Fatty Zucker rats experience mild glucose intolerance, while ZDF become overtly diabetic after 8 wk of age, if they are fed with a diet containing 6.5% of fat. When administered in the fatty Zucker rats, FE 999011 produced a dose-depended reduction in plasma glucose excursion during the OGTT. During an intraduodenal glucose tolerance test it increased GLP-1 levels, while glucose excursions were indistinguishable from that of lean controls. Chronic treatment with FE 999011 in the fatty Zucker rats significantly improved glucose tolerance, as suggested by the decrease in the insulin-to-glucose ratio. Chronic treatment with FE 999011 twice daily in ZDF rats maintained euglycemia for at least 21 d and delayed the onset of diabetes. Lower basal insulin secretion due to improved insulin sensitivity was reported. It also increased basal GLP-1 levels, stabilized food and water intake to prediabetic levels, reduced hypertriglyceridemia and prevented the rise of circulating non-esterified fatty acids (NEFAs). Up-regulation of pancreatic GLP-1 receptor gene expression was also induced by FE 999011.

The DDP-4 inhibitor isoleucine thiazolidine (P32/98) was orally administered for 3 wk to fatty Zucker rats with incipient IGT (iIGT) and 6 wk in rats with manifest IGT (mIGT) in a dose of 21.61 mg/kg (n = 10 per group)[65]. Control rats received the same amount of placebo. Blood glucose day-night profile was significantly reduced in iIGT Zucker rats achieving values near normalization; it was also improved in mIGT rats. P32/98 tended to reduce food intake and body weight gain, as well as non-fasting plasma insulin levels, only in Zucker rats with iIGT. P32/98 bolus before OGTT increased insulin secretion and reduced glucose load both in iIGT and mIGT Zucker rats, suggesting a broad therapeutic efficacy in animal models of IGT. Treatment of isolated pancreatic islets of mIGT Zucker rats with this agent decreased pancreatic insulin content and increased glucose responsiveness, while the β-cell volume density was not improved.

The DPP-4 inhibitor PFK 275-055, a vildaglitpin analogue, was investigated in obese, insulin resistant prediabetic rats for 4 wk in a dose of 10 mg/kg per day[66]. GLP-1 levels increased after PFK 275-055 therapy. Insulin levels were decreased after therapy with this agent, while glucose levels were not affected; an increased β-cell/α-cell ratio was observed. The DPP-4 inhibitor DA-1229 improved pancreatic insulin content, β-cell function and delayed the onset of diabetes in young *db/db* mice[67]. Currently, several studies have been launched and are recruiting individuals in order to explore the possible role of alogliptin and saxagliptin in a prediabetic state[68].

GLP-1R AGONISTS IN A PREDIABETIC STATE

Exenatide

Studies organized in animal models: Exenatide is the synthetic form of the naturally occurring exendin-4, a 39-amino-acid peptide hormone secreted by the salivary glands of the venomous lizard Heloderma suspectum, otherwise known as the Gila monster[69]. It shares 53% structural homology with human GLP-1 and resists inactivation by the DPP-4. In an animal model of profound insulin resistance, IGT, hypertriglyceridemia and decreased β-cell mass, exendine-4 significantly increased β-cell mass by 201%[56]. This effect was achieved after a significant decrease in β-cell apoptosis, although the molecular effect for this activity was not studied. HOMA-IR and HOMA-β indexes remained within normal rage. Normalization of IGT and liver triacylglycerol content was also achieved.

In another well-organized study, exendin-4 was administered to obese prediabetic *db/db* mice at 6 wk of age for 16 d[70]. By the age of 8 wk, vehicle treated mice developed T2D, while mice treated with exendin-4 maintained FPG in the normal range, indicating that this agent delayed the onset of T2D. Improvement in glucose tolerance was also observed with exendin-4. No significant differences were observed between the two groups as far as insulin sensitivity is concerned. Glucose alone induced a two to five-fold increase in insulin secretion in the exendin-4 group, while the pancreas of vehicle-treated mice was unresponsive to the same dose of glucose. A 1.4-fold increase in β-cell mass was observed in exendin-4 mice, which was the result of both increased β-cell proliferation and decreased β-cell apoptosis; these changes were related to higher expression of the protein kinases Akt1 and MAPK.

The ability of exendin-4 to promote β-cell proliferation in young Goto-Kakizaki (GK) rats during the prediabetic state, and therefore prevent the development of T2D when animals become adults, was also explored[71]. Four groups of rats were investigated: two control

groups (control GK and control non-diabetic Wistar rats) and two experimental groups. In the two experimental groups, GK rats received either a subcutaneous daily injection of GLP-1 (400 μg/kg of body weight) or exendin-4 (3 μg/kg of body weight) for five days (day's two to six) after their birth. Animals were killed seven days or two months after birth. Seven days after their birth GK rats showed significantly higher pancreatic insulin content and doubling of β-cell mass compared to the untreated GK group; this effect resulted from both differentiation (neogenesis) and proliferation enhancement of β-cells. Follow up from day seven to the adult age (two months) showed that both treatments decreased postabsorptive basal plasma glucose levels and increased pancreatic insulin content compared to the untreated GK arm. In GK/GLP-1 and GK/exendin-4 groups, β-cell mass was significantly increased and represented 71% and 63% of the β-cell mass of the Wistar group, respectively. Glucose-stimulated insulin release, as evaluated during an IVGTT, was significantly improved in both treated groups. It was concluded that GLP-1 or exendin-4 treatment limited the prediabetic period and delayed the development of T2D in this animal model of prediabetes.

Exendin-4 activity was explored in a rat model of uteroplacental insufficiency[72]. Intrauterine growth retarded (IUGR) rats experience a progressive decline in β-cell mass weeks before the onset of T2D; hence there is a prediabetic neonatal period, which was investigated. At two weeks, exendin-4 significantly decreased body weight in both IUGR and control pups and this effect persisted into adulthood. It also improved glucose tolerance, which was maintained at 7 wk of age. Interestingly, at three months of age, vehicle-treated IUGR rats developed T2D (their β-cell mass declined by almost 80%) whereas exendin-4 treated IUGR rats had NGT and normal β-cell mass. At 18 months of age, exendin-4 treated IUGR rats were normoglycemic, while all vehicle treated IUGR rats had died. Exendin-4 therapy in IUGR rats at 14 d restored PDX-1 mRNA levels, in concentrations similar to controls; this effect persisted for three months.

Clinical studies: One hundred fifty two obese [average body mass index (BMI): 39.6 ± 7.0 kg/m^2] individuals with NGT or IGT or IFG were randomized to receive either exenatide (n = 73) (10 μg with a 4-wk 5 μg dose titration period) or placebo (n = 79), along with lifestyle modification for 24 wk[73]. Thirty eight individuals (25%) had IFG or IGT. Exenatide-treated individuals lost 5.1 ± 0.5 kg from baseline *vs* 1.6 ± 0.5 kg in the placebo group (treatment difference: -3.3%, P < 0.001). An important percentage of individuals with prediabetes returned to NGT after the end of the period (77% compared to 56% in the placebo group). No significant baseline to end point changes was shown for FPG, A1C and OGTT. Diarrhea was reported by 14% and 3% and nausea by 25% and 4% of the exenatide and placebo groups, respectively. Adverse effects were mild or moderate in severity in most cases. It was concluded that exenatide therapy in addition to lifestyle modification is a promising therapeutic approach for obese prediabetic individuals.

In another non randomized study, 105 individuals with IGT and/or IFG were treated with: (1) Lifestyle modification only (n = 18). Participants were advised to achieve 7% body weight loss over three months and to walk 30 min daily, seven days per week; (2) Pioglitazone 15mg daily and metformin 850mg daily (n = 40); and (3) A triple combination of pioglitazone 15mg daily, metformin 850 mg daily and exenatide 10 mcg twice daily (n = 47)[74]. All individuals who received drug therapy had the same advice on lifestyle intervention. Mean follow-up period was 8.9, 6.9, and 5.5 mo in the three groups respectively. Individuals in the lifestyle intervention group achieved only a slight reduction of body weight (82.3 kg to 80.9 kg). No significant change on insulin sensitivity and β-cell function was observed. In the pioglitazone and metformin group FPG was decreased from 109 mg/dL to 102 mg/dL and mean glucose AUC during OGTT was reduced by 12% (P < 0.001). Insulin sensitivity and β-cell function improved by 42% and 50% respectively, while 14% of the individuals with IGT and 36% of the individuals with IFG reverted to NGT. Interestingly, in the triple therapy group, a robust 109% improvement in β-cell function and a 52% increased in insulin sensitivity was observed, while 59% of the individuals with IGT and 56% of the individuals with IFG reverted to NGT. No patient in both double and triple therapy groups developed T2D.

A 24-wk prospective randomized outpatient clinical trial explored the possible role of exenatide (10 μg twice daily) and metformin (1000 mg twice daily), alone or in combination, on menstrual cyclicity and metabolic and endocrinological parameters in 60 overweight/obese women with polycystic ovary syndrome (PCOS)[75]. Forty two participants (70%), 14 in each arm completed the study protocol. Weight loss was more profound in the exenatide arms compared to metformin (P = 0.003). Combination treatment promoted a dramatic improvement in central adiposity. At the end of the study, the combination arm experienced weight loss of 6 ± 0.5 kg, the exenatide arm 3.2 ± 0.1 kg, and the metformin arm 1.6 ± 0.2 kg. Eighteen women with PCOS had glucose intolerance and 11 of them completed the study. Seven (64%) of them had NGT at the end of the trial (three of three in the combination arm, three of five on the metformin arm and one of three on the exenatide arm). Insulin sensitivity and HOMA-IR were significantly improved in all treatment groups. Insulin secretion, as measured by the corrected insulin response at glucose peak, was significantly reduced in the exenatide and combination arms (P < 0.016). The insulin secretion-sensitivity index increased progressively from metformin arm (232 ± 116) to the exenatide arm (395 ± 112) and the combination arm (516 ± 117) (P < 0.005), suggesting an improved β-cell function with enhanced insulin sensitivity.

The role of exenatide in order to improve postprandial endothelial function in individuals with IGT (n =

16) and patients with recent T2D with optimal glycemic control (n = 12) was investigated in a double-blinded randomized crossover study[76]. Endothelial function was estimated by reactive hyperemia peripheral arterial tonometry (PAT). In individuals with IGT, PAT index tended to increase after exenatide and was higher compared to the placebo period. Exenatide reduced postprandial rises in insulin, glucose and triglycerides concentrations. Postprandial PAT index was inversely correlated only with mean postprandial concentrations of triglycerides, possibly due to the high fat content of the meal administered. Change in postprandial triglycerides after exenatide accounted for 64% of the estimated effect of exenatide on postprandial endothelial function. Exenatide also reduced the postprandial elevation of triglycerides, apolipoprotein B-48, apolipoprotein CⅢ, remnant lipoprotein cholesterol and remnant lipoprotein triglyceride in individuals with IGT (n = 20) and patients with recent onset T2D (n = 15)[77]. These effects were not affected either with statin therapy or by glucose tolerance status. Both studies suggested an additional cardiovascular benefit of this agent beyond the improved glycemic control in this population[76,77]. Another randomized 3-wk head-to-head study examined the effects of exenatide *vs* metformin on microvascular endothelial function in 50 individuals with abdominal obesity and prediabetes[78]. Similar effects of both agents were shown on microvascular endothelial function, vascular activation, oxidative stress and markers inflammation. Exenatide did not demonstrate any beneficial effect on postprandial function in individuals with IGT. It was suggested that the reason for this observation was the administration of a glucose-only meal instead of a high fat meal, which would be expected to increase postprandial triglycerides[76,78].

Liraglutide

Studies organized in animal models: Liraglutide is a long acting analog with 97% homology to human GLP-1. It has an additional 16-carbon fatty acid and a small amino acid-spacer that promotes reversible binding to albumin and enhances resistance to DPP-Ⅳ degradation, providing a half-life of approximately 13 h[79]. The possible role of chronic liraglutide therapy in prediabetic UCD-T2D rats, in order to prevent or delay T2D, was investigated in a well organized study[80]. The UCD-T2D rat model develops polygenic adult-onset obesity and insulin resistance, followed by inadequate β-cell compensation and eventually T2D. UCD-T2D rats develop diabetes in a later age than other animal models of T2D; thus they are highly suitable for diabetes prevention studies[81]. At two months of age male sibling rats were divided in three groups (n = 32 per group): a control group (higher energy intake, body weight and adiposity compared to the other groups), a food-restricted group and a liraglutide group (0.2 mg/kg sc for 15 mo). Restricted rats were food restricted to 9% less energy per kg of body weight compared to the liraglutide group, in order to equalize body weights between these two groups. Half of the animals in each group were killed at 6.5 mo for tissue collection, while the remaining half continued treatment until T2D onset. FPG and A1C were lower in the liraglutide and food-restricted groups. Liraglutide treatment delayed T2D onset by 4.1 ± 0.8 mo compared to controls (P < 0.0001) and by 1.3 ± 0.8 mo compared to restricted animals (P < 0.05). Liraglutide-treated animals had lower fasting plasma triglycerides, glucagon and leptin levels, as well as body fat (despite similar body weight), compared to both groups. Decreased body fat could be the result of an increased lipid oxidation. Rats in the liraglutide group had significantly lower fasting plasma insulin compared to the other groups (P < 0.001), starting from one month and lasting throughout the 6 mo period, suggesting that this effect was not solely related to reduced body weight. Liraglutide treatment and energy restriction equally preserved pancreatic insulin content and islet morphology, possibly due to the lower weight gain and delayed hyperglycemia. Pancreatic insulin content in the control group was approximately one-third of that of the two other groups.

In another study, 12-wk old Otsuka-Long-Evans-Tokushima fatty (OLETF) rats (n = 8) were treated with three doses of liraglutide (50, 100, and 200 μg/kg twice a day) or 0.9% saline intraperitoneally (n = 8), twice daily for 12 wk. Eight Long-Evans-Tokushima-Otsuka rats with saline injection served as normal controls[82]. At the end of the 12 wk of treatment, all rats were euthanized and pancreatic tissues were used for histopathological and immunohistochemical analysis; only in the liraglutide 100 μg/kg group an analysis was performed, since this dose can be converted to a human equivalent dose. OLETF rats experienced obesity, IFG, hyperinsulinemia, insulin resistance, increased cholesterol levels, and a high inflammatory state. Although liraglutide treatment had only an acute effect on food intake, its beneficial effect on weight loss was sustained independently of feeding. All three doses of liraglutide suppressed IFG, IGT and insulin resistance. At the end of the 12-wk intervention period, 87.5% of the vehicle-treated OLETF progressed to T2D. On the contrary, 42.9% of IFG rats were reversed to NGT, while none of the liraglutide-treated OLETF rats progressed to T2D compared to vehicle-treated animals (P < 0.0001). Liraglutide improved both triglyceridemia and the inflammatory state observed. It also preserved islet morphology. Up-regulation of the anti-apoptotic Bcl-2 protein and down-regulation of the pro-apoptotic Bax factor were reported, which may contribute to the improvement of pancreatic islet function and structure.

When liraglutide was administered in a dose of 150 mg/kg twice daily for 6 wk in prediabetic rats, it strongly attenuated T2D development[83]. Approximately 53% of the antihyperglycemic effect observed was mediated by a reduction in food intake. In the experiments with 60% pancreatectomized rats, liraglutide significantly reduced glucose excursions after an OGTT. Furthermore, when NGT status was established, no increase in β-cell proliferation and mass was observed in both models of

β-cell deficiencies. It was suggested that the influence of GLP-1 agonism on β-cell mass dynamics *in vivo* was strongly related to the glycemic state observed.

Clinical studies: In a 20-wk prospective multicentre study, 564 nondiabetic obese individuals (31% of whom had prediabetes) were randomized to receive either one of four doses of liraglutide (1.2 mg, 1.8 mg, 2.4 mg, or 3.0 mg, *n:* 95, 90, 93 and 93, respectively) or placebo (n = 98) administered once daily subcutaneously or open label orlistat 120 mg three times daily (n = 95)[84]. All individuals increased their physical activity using pedometers and were advised to adhere a low fat diet with about to 500 kcal per day deficit. Sixty-one percent of the individuals in the liraglutide groups lost at least 5% of body weight from baseline, which was significantly more than the placebo arm. The proportion of individuals who lost more than 10% of baseline weight was dose depended and was greater in the 3 mg liraglutide arm than in the placebo arm (28% *vs* 2%). Systolic/diastolic blood pressure was reduced by 5.7/3.7 mmHg. The incidence of metabolic syndrome was reduced by more than 60% in those treated with liraglutide 2.4 mg and 3.0 mg. The prevalence of prediabetes was decreased by 84-96% with liraglutide 1.8 mg, 2.4 mg and 3 mg. Mean FPG was decreased by 7%-8% in the liraglutide arm, while no visible effect was described in the two other arms. Mean A1C was slightly reduced in a dose depended fashion in individuals treated with liraglutide compared to that in the two other groups. Mean change in plasma glucose during OGTT was reduced in all liraglutide groups compared to that of orlistat and placebo. Liraglutide therapy did not have any effect on insulin resistance as estimated by HOMA. However, median β-cell function was decreased with orlistat and placebo by 21% and 17% respectively, but increased in the liraglutide arm by 5%-24%. Fasting insulin levels initially increased, but as body weight and glucose concentrations gradually decreased, insulin levels were reduced, suggesting the glucose-depended activity of liraglutide on insulin secretion.

The two-year results from the extension of this 20-wk trial were recently reported[85]. Three hundred ninety eight individuals entered the extension and 268 (67%) completed the two-year trial. All participants continued on randomization treatment for one year, after which liraglutide or placebo individuals switched initially to liraglutide 2.4 mg and then 3 mg (based on 20-wk and one-year results, respectively). After two years, individuals on liraglutide 2.4/3.0 mg lost 3.0 kg (1.3-4.7 kg) more weight than those on orlistat (P < 0.001). Approximately 70% of the individuals on liraglutide 2.4/3.0 mg maintained weight loss more than 5% of screening weight after two years, 43% maintained more than 10% loss and 25% maintained more than 15% loss. Estimated weight loss of 7.8 kg and mean systolic blood pressure reduction of 12.5 mmHg was sustained with liraglutide 2.4/3.0 mg in completers from screening. Between 52%-62% of liraglutide-treated individuals with prediabetes at randomization achieved NGT after two years compared to 26% in the orlistat arm. Mean FPG and A1C concentrations were also reduced. The two year prevalence of prediabetes and metabolic syndrome in the liraglutide 2.4/3.0 mg group was decreased by 52% and 59% respectively. The most frequent liraglutide-associated adverse effects were gastrointestinal, mainly nausea and vomiting, as expected from T2D trials. However, most nausea/vomiting episodes were transient; more than 90% were mild or moderate in intensity.

Recently, a 14-wk double blind, randomized placebo-controlled study was launched in order to investigate the possible role of liraglutide 1.8 mg treatment in 68 older (mean age: 58 ± 8 years) overweight/obese (mean BMI: 31.9 kg/m^2) individuals with prediabetes (IFG and/or IGT)[86]. Participants were also advised to eat a moderate carbohydrate diet and decrease total caloric intake by 500 kcal/d. Twenty four (68%) individuals randomized in the liraglutide group and 27 (82%) individuals in the placebo group completed testing at the end of the trial. Participants randomized to liraglutide arm lost twice as much weight as those assigned to placebo (6.8 kg *vs* 3.3 kg; P < 0.001). More individuals in the liraglutide arm finally lost 7% of baseline weight compared to the placebo arm (54% *vs* 4%); 10% weight loss was only observed in the liraglutide arm (17%). Weight loss after liraglutide therapy was associated with significant reduction of insulin resistance. Steady state plasma glucose concentrations were reduced by 29% in the liraglutide arm compared with no change in the placebo arm; FPG (-0.5 mmol/L *vs* 0 mmol/L), systolic blood pressure (-8.1 mmHg *vs* -2.6 mmHg), and triglyceride levels (-0.4 mmol/L *vs* -0.1 mmol/L) were also significantly decreased in the liraglutide arm compared to the placebo arm respectively ($P \leqslant 0.04$). In addition, 75% of the participants in the liraglutide arm achieved normal FPG. The most common adverse effect in the liraglutide arm was nausea (67% *vs* 26% in the placebo arm). It was suggested that the improvement of glycemia in the liraglutide group appeared to be better than reported with weight loss alone in this population.

Indeed, the effects of GLP-1R agonists on insulin secretion are not a simple phenomenon. These medications can increase glucose secretion in a glucose-depended manner after acting directly on the β-cell; they can also decrease insulin secretion secondary to weight loss and enhancement of insulin sensitivity. In this view, it is unclear what the net effect would be when they are administered in individuals with prediabetes. In order to investigate this observation, a parallel study was organized in order to evaluate the relative impact of the indirect effect of weight loss and increase insulin sensitivity compared to the direct effect of GLP-1R agonists on β-cell function[86,87]. In this recent double-blind, randomized, placebo-controlled, parallel-group study 49 individuals (mean age: 58 years, mean BMI: 32.9 kg/m^2) with prediabetes (isolated IFG, isolated IGT and combined IFG/IGT) received either liraglutide 1.8 mg daily (n = 24) or placebo (n = 25). All participants were instructed

to decrease total energy intake by 500 kcal per day and to continue their baseline physical activity[87]. There was a little overlap in the degree of weight loss between the two arms since 88% of the individuals in the liraglutide arm lost more than 5% of baseline body weight compared to 22% in the placebo arm. Weight loss promoted a significant improvement on insulin resistance in the liraglutide arm compared to the placebo arm (-7.7% *vs* -3.9%, $P < 0.001$). Insulin response, after intravenous glucose infusion, was decreased by 7% in the placebo arm whereas it increased by 34% in the liraglutide arm. C-peptide AUC was increased by 29% in individuals receiving liraglutide and NEFAs concentration was reduced. Placebo treatment had no effect on these two parameters. Regression analyses suggested that weight loss was not associated with any changes in pancreatic β-cell function. Despite weight loss and reduction of insulin resistance in the liraglutide arm, the insulin secretion rate was significantly increased and there was no association between weight loss and changes on insulin secretion. It was concluded that changes following liraglutide treatment in patients with prediabetes are not those that are described after weight loss and improved insulin sensitivity, but rather similar effects after an acute GLP-1 infusion[87,88].

SAFETY OF INCRETIN-BASED THERAPIES

An acceptable safety profile is of major importance for every intervention administered in order to prevent or delay T2D. As far as GLP-1R agonists are concerned, the most common adverse effects are gastrointestinal, including nausea, vomiting and diarrhea[89]. However, they occur early on during treatment and tend to be transient. For DPP-4 inhibitors, adverse effects resemble that of placebo, with nasopharyngitis and headache being the most common described[90]. Moreover, discontinuation of therapy because of side effects was similar to placebo[91].

Small preclinical studies, as well as some post-marketing reports, raised the possibility of an increased risk of pancreatitis with incretin based therapies[92-96]. In a study that data were collected from the Food and Drug Administration (FDA) adverse event reporting system database, GLP-1 based therapies were associated with pancreatitis and pancreatic cancer[97]. Another case-control study reported an increased risk for hospitalization for acute pancreatitis with GLP-1 based therapies (after combining exenatide and sitaglitpin treatments) and adjusting for potential confounders[98]. Concerns were also raised after the results of a study organized in organ donors with T2D, who received either sitagliptin or exenatide. A possible expansion of endocrine and exocrine pancreatic compartments after incretin-based therapy, the former being associated by α-cell hyperplasia with the potential progression to neuroendocrine tumors and the latter with an enhanced proliferation and dysplasia, was described[99]. Furthermore, a recent case-control analysis, based on the French pharmacovigilance database, suggested an association of all incretin-based therapies with pancreatitis[100]. A trend towards a slightly elevated risk of pancreatitis, only with GLP-1R agonists, was also shown in a recent pooled analysis of phase Ⅲ trials, although the number of cases was very small and the statistical power was limited[101].

However larger preclinical studies did not established an association of incretin-based therapies with pancreatitis[102-109]. Interestingly in three of these studies, GLP-1R activation or DPP-4 inhibition had a beneficial effect on exocrine pancreatic function and structure[103,104]. A recent study also suggested that pancreatic findings attributed to incretin-based therapies in rodents are commonly observed background findings, without any drug treatment and independent of diet or glycemic status[110]. Moreover large retrospective population studies and recent meta-analysis suggested a negative association of incretin-based therapies with either pancreatitis or pancreatic cancer[111-120]. Recently the FDA reevaluated more than 250 toxicology studies, organized in nearly 18000 healthy animals, and found no association with pancreatitis or any pancreatic toxicity. The European Medicines Agency conducted a same review and reported no pancreatic tumors in mice and rats treated with incretin-based drugs, even at doses that greatly exceed the level of human clinical exposure[121].

A higher expression of GLP-1Rs in rodent calcitonin-producing thyroid C cells, (mainly in rats and mice) combined with sustained GLP-1R activation can result in stimulation of calcitonin secretion, hyperplasia, adenoma and eventually medullary thyroid cancer[122,123]. Indeed, both liraglutide and exenatide were shown to promote the development of thyroid C cell cancer after chronic therapy in rodents[122]. An elevated risk for thyroid carcinoma was described in one study[97]. However, thyroid C cells in humans and monkeys express lower levels of GLP-1Rs[124]. Long-term treatment with high doses liraglutide did not produced thyroid C cell proliferation in monkeys, while no association between calcitonin levels and liraglutide, up to 3 mg daily, was established in large numbers of patients with T2D[125].

Retrospective analysis of phase Ⅲ clinical trials, in which major cardiovascular events were reported as adverse events, have been published for exenatide, liraglutide, vildagliptin, sitagliptin, alogliptin, saxagliptin, and linagliptin[126]. In all of these studies the relative risk for a major cardiovascular event (acute myocardial infarction, stroke and cardiovascular death) was reduced relative to placebo or a comparator therapy to a value below one. However, the 95%CI was more than one in most of these studies, thus the number of events was too small so as to extract definite conclusions. Both the Saxagliptin Assessment of Vascular Outcomes Recorded (SAVOR)-Thrombolysis in Myocardial Infarction (TIMI) 53 (SAVOR-TIMI 53) and the Examination of Cardiovascular Outcomes with Alogliptin *vs* Standard of Care (EXAMINE) trials met the FDA criteria for non inferiority of saxagliptin and alogliptin over placebo respectively, but unfortunately they did not demonstrated any positive evi-

Table 1 Main clinical studies of dipeptidyl peptidase-4 inhibitors in a prediabetic state

Ref.	Study population	Study design	Main results
Utzschneider *et al*[50]	22 individuals with IFG	VILDA was administered in a dose of 100 mg daily for 6 wk. Two weeks of placebo treatment before (running period) and after (washout period) 6 wk was studied	FPG levels were not significantly reduced. AUC GLU and 2-h GLU decreased after a MTT. DI was increased by 69% and insulin sensitivity by 25% after an IVGTT These effects were not sustained in the washout period
Rosenstock *et al*[51]	179 individuals with IGT (80%: IFG + IGT)	Multicenter 12-wk double-blind study 90 participants received VILDA 50 mg/daily and 89 received placebo therapy	Improvements in β-cell function as estimated by insulin secretion relative to that of GLU. Improvements were also reported in α-cell function. These beneficial effects contributed to approximately 30% reduction in prandial GLU excursions
Werzowa *et al*[52]	48 IGT renal transplant recipients	3-mo, double-blind, placebo-controlled study. Participants were randomized to receive 50 mg of VILDA, 30 mg of PIO or placebo in a 1:1:1 ratio (n = 16 in each arm)	A1C reduction was statistically significant between treatment groups and placebo. VILDA and PIO reduced the 2 h plasma GLU at three months compared with baseline, while only PIO reduced FPG
Bock *et al*[57]	22 individuals with IFG	8-wk double blind placebo-controlled study Participants received SITA 100 mg daily (n = 11) or placebo (n = 11)	SITA increased postprandial intact GLP-1 concentrations. Both fasting and postprandial GLU values were unchanged with SITA therapy. A slightly increased DI was reported
Perreault *et al*[58]	23 individuals with either IFG (n = 10) or NGT (n = 13)	4-wk open-label, parallel group study. All participants received SITA 100 mg once daily	SITA resulted in a small, but significant decrease in FPG compared to baseline in both groups (P < 0.05) Administration of SITA did not altered insulin or GLU excursions in the post-intervention OGTT, but did increase AUC for active GLP-1 and C-peptide compared to baseline levels (P < 0.01 for both)

GLP-1: Glucagon-like peptide 1; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; FPG: Fasting plasma glucose; AUC: Area under the curve; DI: Disposition index; IVGTT: Intravenous glucose tolerance test; MTT: Meal tolerance test; A1C: Glycated hemoglobulin; VILDA: Vildagliptin; SITA: Sitagliptin; PIO: Pioglitazone; GLU: Glucose; OGTT: Oral glucose tolerance test.

dence on cardiovascular risk reduction[127,128]. Two recent meta-analysis suggested that DPP-4 inhibitors may have a neutral effect or reduce the risk of cardiovascular events and all-cause mortality in patients with T2D[129,130]. As far as GLP-1R agonists are concerned two recent meta-analysis reported that these agents do not appear to increase cardiovascular morbidity in comparison with placebo or other active drugs[131,132].

Hospitalization for heart failure among T2D who received saxagliptin in the SAVOR-TIMI 53 was increased by 27% compared to the placebo group (3.5% *vs* 2.8%; HR = 1.27; 95%CI: 1.07-1.51; P = 0.007), while no association of alogliptin with heart failure was found in the EXAMINE study[133]. Two recent meta-analysis suggested a possible increased risk of developing heart failure after DPP-4 therapy[134,135]. Currently, a large number of long-term cardiovascular outcome trials in patients with T2D are being performed in order to clarify the cardiovascular safety and efficacy of incretin-based therapies[136].

In addition to safety and efficacy of incretin-based therapies, cost is another significant issue that must be taken into consideration. Although the cost of incretin-based therapies is greater compared to other glucose-lowering therapies, long term effectiveness of these agents can be associated with a decreased in the cost of management of T2D and its complications compared to other therapies[137].

CONCLUSIONS-PERSPECTIVES

During the last two decades there has been an immense investigation in order to understand the pathophysiology of the early stages of hyperglycemia, which very often progress to overt T2D within a few years, as β-cell decline and failure progresses. The huge burden resulting from the complications of T2D created the need of novel therapeutic strategies in an effort to prevent its development[8]. The beneficial effects of incretin-based therapies on β-cell function in patients with T2D, together with their strictly glucose-depended mechanism of action, suggested their possible use in individuals with prediabetes, when greater β-cell mass and function are preserved and the possibility of β-cell salvage is higher[138]. The main results of the most important clinical studies of incretin-based therapies in individuals with prediabetes are shown in Tables 1 and 2.

DPP-4 inhibitors have shown beneficial effects on β-cell mass and function in preclinical models of prediabetes. However short-term clinical studies (maximum duration of 12 wk) have only demonstrated a modest effect on glucose homeostasis, which was lost after treatment discontinuation[50]. Whether longer periods of DPP-4 inhibition in individuals with prediabetes can measurably alter β-cell function, in a way that is sustained even after treatment discontinuation, remains unproven. One year treatment with vildagliptin in drug-naïve patients with T2D and mild hyperglycemia initially increased β-cell secretory capacity, but this effect was not maintained after the washout period[139]. However, when vildagliptin was administered in drug-naïve patients with T2D and mild hyperglycemia (A1C: 6.2%-7.2%) for two years, β-cell function tended to be greater after two years than after

Table 2 Main clinical studies of glucagon-like peptide-1 receptor agonists in a prediabetic state

Ref.	Study population	Study design	Main results
Rosenstock *et al*[73]	152 obese individuals of whom 38 had IGT or IFG	Participants were randomized to receive either EXE (n = 73) (10 μg with a 4-wk 5 μg dose titration period) or placebo (n = 79) along with lifestyle modification for 24 wk	EXE-treated individuals lost 5.1 ± 0.5 kg from baseline *vs* 1.6 ± 0.5 kg in the placebo group (P < 0.001). An important percentage of individuals with prediabetes returned to NGT after the end period (77% compared to 56% in the placebo group)
Armato *et al*[74]	105 individuals with IGT and/or IFG. Mean follow-up period was 8.9, 6.9, and 5.5 mo in the three groups respectively	Participants were treated with: (1) Lifestyle modification only (n = 18); (2) PIO 15 mg daily and MET 850 mg daily (n = 40); and (3) PIO 15 mg daily, MET 850 mg daily and EXE 10 mcg twice daily (n = 47)	A robust 109% improvement in β-cell function and 52% increased in insulin sensitivity was observed in the EXE group, while 59% of individuals with IGT and 56% individuals with IFG reverted to NGT. No patient in both double and triple therapy groups developed T2D
Astrup *et al*[84]	564 obese individuals (31% had prediabetes)	20 wk double-blind prospective multicentre study. Participants were randomized to receive either one of four doses of LIRA (1.2 mg, 1.8 mg, 2.4 mg, or 3.0 mg, n: 95, 90, 93 and 93) or placebo (n = 98) or open label orlistat 120 mg three times a day (n = 95)	61% of the individuals in the LIRA groups lost at least 5% of body weight from the baseline, which was significantly more than in the placebo arm. The prevalence of prediabetes was decreased by 84%-96% with LIRA 1.8 mg, 2.4 mg and 3 mg. Mean FPG was decreased by 7%-8% only in the LIRA arm. Mean change in plasma GLU during OGTT were reduced in all LIRA groups compared with that of orlistat and placebo. Median β-cell function increased in the LIRA arm by 5%-24%
Kim *et al*[86]	68 overweight/obese individuals with IFG and/or IGT	14 wk double blind randomized placebo-controlled study. 24 individuals received LIRA 1.8 mg daily and 27 placebo therapy	Participants randomized to LIRA arm lost twice as much weight as those assigned to placebo (P < 0.001). Steady sate plasma GLU was reduced by 29% in the LIRA arm compared with no change in the placebo arm. 75% of the participants in the LIRA arm achieved normal FPG
Kim *et al*[87]	49 individual with isolated IFG, isolated IGT and combined IFG/IGT	14 wk double-blind, randomized, placebo-controlled, parallel-group study. Participants received LIRA 1.8 mg daily (n = 24) or placebo (n = 25)	Weight loss promoted a significant improvement in insulin resistance in the LIRA arm compared to the placebo arm (-7.7% *vs* -3.9%, P < 0.001). Insulin response, after intravenous GLU infusion, was decreased by 7% in the placebo arm whereas it increased by 34% in the LIRA arm. Despite weight loss and reduction of insulin resistance in the LIRA arm, the insulin secretion rate was significantly increased and there was no association between weight loss and changes in insulin secretion

IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; FPG: Fasting plasma glucose; EXE: Exenatide; LIRA: Liraglutide; PIO: Pioglitazone; MET: Metformin; T2D: Type 2 diabetes; GLU: Glucose; OGTT: Oral glucose tolerance test.

one year of treatment[140].

GLP-1R agonists have also shown significant improvements on β-cell mass and function in preclinical studies. Important improvements on β-cell function and insulin sensitivity were also reported in short term clinical studies, in which an important percentage of individuals with prediabetes returned to NGT. Weigh reduction in overweight and obese individuals with prediabetes was also shown, as well as improvements of endothelial function and lipid profile. Whether GLP-1R agonists can prevent or delay the transition to T2D needs further investigation in well-designed long term studies. The Restoring Insulin Secretion consortium will examine whether medication, including liraglutide, or surgical intervention strategies can reduce the progressive β-cell dysfunction in adults and youth with prediabetes or early T2D[141]. The duration of GLP-1R agonists therapy in order to promote sustained β-cell improvements is also an issue of investigation. Interestingly, when exenatide was administered in patients with T2D for one year, the treatment related improvement of β-cell function was lost after a four-week drug cessation[142]. However, the three-year data of exenatide treatment suggested a small but statistically significant effect on DI following a four-week off therapy period[143].

Recent evidence also demonstrates the presence of genetically induced GLP-1 resistance both in prediabetic and diabetic states. Whether pharmacogenomic studies are needed in order to identify responders and non-responders to incretin based therapies regarding glucose metabolism, is an issue of future research[144].

The safety of incretin-based therapies remains a topic of scientific discussion and exploration[126,145,146]. Currently, precise estimates for the risk of possible serious adverse effects associated with incretin-based therapies cannot be estimated. Future data from cardiovascular outcome studies and ongoing clinical studies, which will improve the statistical power of prospective studies and facilitate larger meta-analyses, are crucially anticipated in order to clarify their long-term safety. Until these data are available, large, long term, well designed future diabetes prevention trials of incretin-based therapies will be required in order to determine whether they can stabilize or reverse β-cell loss and promote a sustained reduction in the development of T2D in this population.

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P- Reviewer: Cheng TH, Efstathiou S, Martinez-Costa OH
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Adipokines as a novel link between obesity and atherosclerosis

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Received: December 15, 2013 Revised: April 14, 2014
Accepted: May 15, 2014
Published online: June 15, 2014

Abstract

The traditional perception of adipose tissue as a storage organ of fatty acids has been replaced by the notion that adipose tissue is an active endocrine organ, releasing various adipokines that are involved in the pathogenesis of obesity-related metabolic disturbances. Obesity is a well-known risk factor for atherosclerosis, and accelerates atherosclerosis by many mechanisms such as increase in blood pressure and glucose level, abnormal lipid profiles, and systemic inflammation. Furthermore, growing evidence suggests that some adipokines directly mediate the process of atherosclerosis by influencing the function of endothelial cells, arterial smooth muscle cells, and macrophages in vessel walls. In obese patients, the secretion and coordination of such adipokines is abnormal, and the secretion of specific adipokines increases or decreases. Accordingly, the discovery of new adipokines and elucidation of their functions might lead to a new treatment strategy for metabolic disorders related to obesity, including cardiovascular diseases.

Key words: Adipose tissue; Adipokine; Obesity; Atherosclerosis; Inflammation

Core tip: This review summarizes recent laboratory and clinical studies on the influence of various adipokines, including adiponectin, resistin, adipocyte fatty acid binding protein, omentin-1, and chemerin, on the development of atherosclerosis.

Original sources: Yoo HJ, Choi KM. Adipokines as a novel link between obesity and atherosclerosis. *World J Diabetes* 2014; 5(3): 357-363 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/357.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.357

INTRODUCTION

Obesity is an important risk factor for atherosclerosis, but the underlying mechanism for this association is poorly understood. Adipose tissue was considered to be a store of surplus energy, but is now recognized as an independent and active endocrine organ. Various adipokines, such as leptin (a protein secreted by fat cells), tumor necrosis factor-α (TNF-α), resistin, and adiponectin significantly affect obesity-related metabolic diseases by controlling fat metabolism, energy homeostasis, and insulin sensitivity[1]. Independent of their effects on glucose and fat metabolism, some adipokines have been regarded recently as direct links between obesity and atherosclerosis because of their influence on the function of endothelial cells, arterial smooth muscle cells, and macrophages in vessel walls[2] (Figure 1). The identification of a novel adipokine that regulates the atherosclerotic process might provide new opportunities for developing more effective approaches for preventing cardiovascular disease. This review will focus on adipokines that mediate obesity and atherosclerosis, including adiponectin, resistin, adipocyte fatty acid binding protein (A-FABP), omentin-1, and chemerin.

ADIPONECTIN

Adiponectin was the first 30-kDa protein cloned from fat

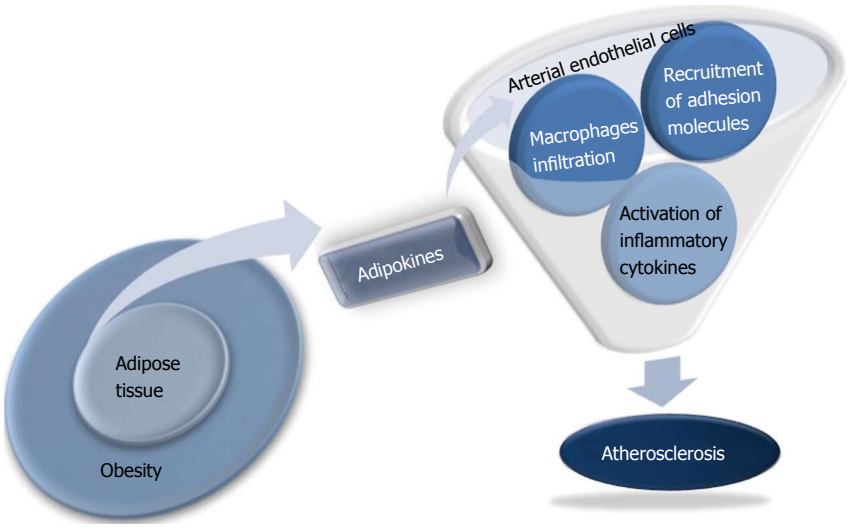

Figure 1 Novel function of adipokines as a direct link between obesity and atherosclerosis.

tissues[3]. Adiponectin is a metabolically active adipokine that is inversely associated with obesity, insulin resistance, and atherosclerosis[4,5]. Adiponectin promotes fatty acid oxidation through the phosphorylation of 5-AMP-activated protein kinase (AMPK), thereby stimulating acetyl-CoA carboxylase. The adiponectin receptors AdipoR1 and AdipoR2 are responsible for adiponectin signaling and biological function. Yamauchi *et al*[6] reported that insulin resistance occurred in AdipoR1/R2 knockout mice, but when AdipoR1 or AdipoR2 were overexpressed in the liver by using adenovirus, glucose metabolism improved in terms of increase in AMPK vitality and peroxisome proliferator-activated receptors α expression. Adiponectin is a metabolically active adipokine which has anti-inflammatory, antiatherogenic, and antidiabetic properties[7] and is therefore inversely associated with obesity, insulin resistance, and atherosclerosis. Hypoadiponectinemia has been established as an independent risk factor for type 2 diabetes and cardiovascular disease (CVD)[8]. We previously showed that, after adjusting for age, sex, obesity, history of impaired fasting glucose or impaired glucose tolerance, hypertension, and dyslipidemia, lower baseline serum adiponectin concentrations are associated significantly with the development of type 2 diabetes and metabolic syndrome[9]. On the other side, the Health Professionals Follow-Up Study showed that high plasma adiponectin levels were associated with a lower risk of myocardial infarction in men during 6 years of follow-up studies[10].

Experimental studies have shown that adiponectin plays a protective role against the development of inflammation and atherosclerosis. Ouchi *et al*[11] demonstrated that adiponectin specifically suppressed TNF-α-induced nuclear factor κ light chain enhancer of activated B cells (NF-κB) activation in human aortic endothelial cells (HAECs) through a cAMP-dependent pathway. Furthermore, adiponectin suppressed TNF-α-mediated induction of adhesion molecule expression in HAECs. Recently, we reported that serum adiponectin levels had a significant negative correlation with vascular inflammation as indicated by the mean target to background ratio (TBR), suggesting a cardio-protective effect of adiponectin[12].

RESISTIN

Resistin was originally discovered as an adipokine with a possible link between obesity and insulin resistance in rodents[13]. In contrast to rodents, human resistin is expressed primarily in inflammatory cells and has been shown to be involved in obesity-related subclinical inflammation, atherosclerosis, and CVD[14]. Reilly *et al*[15] showed that circulating resistin levels are correlated with inflammation markers and are predictive of coronary atherosclerosis, as measured by coronary artery calcification scores, independent of C-reactive protein. Kawanami *et al*[16] found that resistin induces the expression of adhesion molecules, such as vascular cellular adhesion molecule-1 and intercellular adhesion molecule-1 and that adiponectin inhibit the effect of resistin in vascular endothelial cells. Lee *et al*[17] observed that resistin promotes foam cell formation *via* the dysregulation of scavenger receptors macrophages. In men with acute myocardial infarction, a multivariate model revealed that obesity and C-reactive protein were independent variables associated with higher resistin levels[18]. In a cross-sectional study of 3193 Chinese subjects, resistin was more significantly associated with fibrinolytic and inflammatory markers than with obesity or insulin resistance[19]. Moreover, Weikert *et al*[20] reported that individuals in the highest quartile of resistin levels had a significantly increased risk of myocardial infarction compared with those in the lowest quartile of resistin levels after adjustment for cardiovascular risk factors, including C-reactive protein (RR = 2.09; 95%CI: 1.01-4.31) in 26490 middle-aged subjects. Among 397 South Korean patients with acute myocardial infarction, high resistin level was an significant predictor for all-cause mortality, independent of other confounding risk factors[21]. We also showed that serum resistin levels were positively correlated with vascular inflammation mea-

sured using ^{18}F-fluoro-deoxyglucose positron emission tomography[12]. These studies suggest that resistin may represent a novel linkage of metabolic signals, inflammation, and atherosclerosis.

ADIPOCYTE FATTY ACID BINDING PROTEIN

A-FABP is a cytoplasmic protein that combines with saturated and unsaturated fatty acids to control the distribution of fatty acids in various inflammatory response and metabolic pathways[22]. Since Xu *et al*[23] established that the serum concentration of A-FABP, which is synthesized in cytoplasm and secreted into serum, is significantly correlated with components of metabolic syndrome, the role of A-FABP in metabolic syndrome has been studied with renewed interest. Uysal *et al*[24] proved through an oral glucose tolerance test that insulin sensitivity was increased in A-FABP knock out ob/ob mice compared with control mice. In prospective studies, circulating A-FABP has been shown to predict the development of metabolic syndrome and type 2 diabetes independent of adiposity and insulin resistance[25,26].

A-FABP has been shown to be a major mediator of vulnerable plaque formation in various animal and *in vitro* studies. The survival rates of apoE$^{-/-}$ mice null for both A-FABP and mal1 were significantly higher than apoE$^{-/-}$ control mice, primarily because of increased stability of atherosclerotic plaques[27]. In macrophage cell lines, adenovirus-mediated over-expression of A-FABP directly induced foam cell formation by increasing intracellular lipid accumulation, which is an essential step in the formation of atherosclerotic plaques[28]. In contrast, A-FABP$^{-/-}$ macrophages displayed significantly decreased intracellular cholesterol ester accumulation *in vitro*[29] and suppressed production of inflammatory cytokines, such as TNF-α, monocyte chemoattractant protein-1, and interleukin (IL)-6, compared with wild-type controls[30]. Furthermore, Furuhashi *et al*[31] reported that an orally active small molecule inhibitor of A-FABP was an effective therapeutic agent against severe atherosclerosis in mouse models. Recently, a few clinical studies have shown that circulating A-FABP levels are closely related to the development of atherosclerosis in humans. In Korean subjects in whom coronary angiograms were performed for evaluation of chest pain, serum A-FABP levels increased as the number of stenotic coronary arteries increased[32]. Serum A-FABP was shown to be independently associated with carotid intima-media thickness (IMT) in Chinese women after adjusting for other risk factors, including age, obesity, and blood pressure[33]. In patients with coronary artery disease recruited to undergo elective percutaneous coronary intervention, Miyoshi *et al*[34] showed that increased serum A-FABP levels were significantly associated with a greater coronary plaque burden as quantified by intravascular ultrasound. After adjusting for other cardiovascular risk factor in South Korean men without cardiovascular disease or diabetes, we reported that circulating A-FABP levels were independently associated with vascular inflammation as measured by maximum TBR values[35], suggesting A-FABP as a promising key link between different metabolic pathways of adiposity and inflammation.

OMENTIN-1

Omentin is a visceral fat-specific adipokine discovered through expressed sequence tag analysis[36] that has paracrine and autocrine roles in improving insulin sensitivity. Yang *et al*[37] demonstrated that the addition of recombinant omentin stimulated glucose uptake in human adipocytes *via* the activation of Akt phosphorylation. Recent studies showed that omentin increased insulin signal transduction and that it was significantly negatively correlated with metabolic risk factors, including obesity and hyperglycemia, thereby suggesting a beneficial role in energy homeostasis[38-40]. In human clinical studies, it has been suggested that serum omentin-1 levels were significantly decreased in metabolically unhealthy states, such as metabolic syndrome, types 2 diabetes mellitus, and polycystic ovarian syndrome[38-40].

Expression of the omentin gene in interstitial and endothelial cells suggests multi-functionality[41,42]. Fain *et al*[43] were the first to demonstrate the predominant expression of omentin mRNA in human epicardial fat, suggesting that omentin might influence coronary atherogenesis like other periadventitial epicardial adipokines. Some researchers reported that omentin might modulate vascular function through direct action on endothelial cells[44,45]. The vasodilating effect of omentin on isolated rat aorta, mediated by endothelium-derived nitric oxide, was first examined by Yamawaki *et al*[45]. Treatment of human endothelial cells with omentin prevented TNF-α-induced cyclooxygenase-2 expression by inhibiting c-Jun N-terminal kinase signaling, suggesting an anti-inflammatory function of omentin on endothelial cells[44]. Recently, several *in vivo* studies that might explain the mechanism underlying the connection between circulating omentin-1 and the atherosclerotic process have been published. In human endothelial cells, omentin significantly decreased C-reactive protein and TNF-α-induced NF-κB[46]. Xie *et al*[47] reported that adenovirus-mediated overexpression of omentin-1 attenuated arterial calcification in OPG$^{-/-}$ mice, suggesting that increasing concentrations of omentin-1 might be beneficial by protecting arteries. In an *in vitro* study, treatment of calcifying vascular smooth muscle cells (CVSMs) with omentin inhibited osteoblastic differentiation of CVSMCs *via* the phosphatidylinositol 3-kinase/Akt signaling pathway[48]. Very recently, Maruyama *et al*[49] reported that systemic delivery of an adenoviral vector expressing omentin enhanced blood flow recovery and capillary density in ischemic limbs of wild type mice. Taken together, these *in vitro* data suggest the possibility that lower omentin levels contribute to the development of cardiovascular disease from initiating early endothelial dysfunction to arterial calcification.

There have been many clinical studies examining the

significance of correlations of circulating omentin-1 levels with brachial artery vascular reactivity, carotid intima media thickness, and coronary heart disease[50-53]. Moreno-Navarrete *et al*[50] demonstrated that the concentration of circulating omentin-1 contributes independently to endothelial dysfunction, even after controlling for adiposity, age, and inflammation in subjects with impaired glucose tolerance. In that study, vascular reactivity was measured using high-resolution ultrasound imaging of the brachial artery. Subsequently, two reports on the negative correlation of serum omentin-1 with carotid IMT have suggested cardioprotective and anti-atherosclerotic roles for omentin-1[52,53]. Liu *et al*[53] demonstrated that the serum omentin-1 level was independently correlated with carotid IMT in metabolic syndrome patients, and Shibata *et al*[52] showed similar results in apparently healthy men. Recently, El-Mesallamy *et al*[54] examined the level of circulating omentin-1 in an Egyptian population with type 2 diabetes, with or without ischemic heart disease. Although they did not detect clear differences in serum omentin-1 levels between patients with type 2 diabetes with or without ischemic heart disease, multiple regression analysis showed that the IL-6 level was an independent risk factor influencing serum omentin-1 level. This suggests that omentin-1 is regulated by inflammation. Therefore, omentin is regarded as a novel link between insulin resistance, inflammation, and cardiovascular disease, suggesting its possibility as a novel therapeutic target for the cardiovascular diseases.

CHEMERIN

Chemerin was identified as a chemoattractant that promotes the recruitment of immature dendritic cells and macrophages to lymphoid organs and sites of tissue injury[55]. Goralski *et al*[56] first reported a high level of chemerin expression in mouse and human adipocytes. They also reported that loss of chemerin expression almost completely abrogated adipogenesis in 3T3-L1 cells, and modified the expression of genes important in glucose and lipid metabolism, such as GLUT4, leptin, and adiponectin[56]. After that, Ernst *et al*[57] reported that exogenous administration of chemerin exacerbated glucose intolerance, lowered serum insulin levels, and decreased tissue glucose uptake in a mouse model of obesity and diabetes. Growing evidence from human data supports a linkage between chemerin, obesity, and metabolic syndrome. A study of a Mexican-American population showed that circulating chemerin levels were significantly higher in obese subjects compared with non-obese controls. Plasma levels of chemerin were correlated positively with body mass index (BMI), fasting glucose, fasting insulin, and triglycerides levels, and negatively correlated with high-density lipoprotein (HDL)-cholesterol level[58]. Bozaoglu *et al*[58,59] demonstrated that serum chemerin levels were closely correlated with BMI, fasting serum insulin, triglycerides, and HDL-cholesterol in non-diabetic subjects. Sell *et al*[60] reported that in patients who had undergone bariatric surgery for weight reduction, the serum chemerin levels were significantly reduced after surgery, indicating that chemerin might mediate the metabolic alterations in obesity.

Although chemerin is a well-known secreted protein with an established role in immune function, recent experimental data indicate that chemerin might provide a link between obesity and chronic inflammation[61]. Recently, Sell *et al*[62] reported that chemerin activated the NF-κB pathway and impaired glucose uptake in primary human skeletal muscle cells. Moreover, TNF-α treatment of 3T3-L1 adipocytes increased bioactive chemerin levels, suggesting that inflammatory cytokines contribute to the up-regulation of chemerin in obesity[63]. Thus, adipocyte-derived chemerin might be involved in the pathogenesis of obesity-related inflammatory disorders, including atherosclerosis. Although Becker *et al*[64] showed that the expression of chemerin did not significantly alter the extent of atherosclerosis in low-density lipoprotein cholesterol receptor knockout mice, they hypothesized that chemerin might affect early atherosclerotic plaque development and morphology rather than the extent of the atherosclerotic lesion area. Hart *et al*[65] showed that chemerin rapidly stimulated the adhesion of macrophages to the extracellular matrix protein, fibronectin, and to the adhesion molecule, vascular cell adhesion molecule-1, suggesting that chemerin might promote the progression of atherosclerosis. Furthermore, Kaur *et al*[66] demonstrated the novel presence of a G-protein coupled chemerin receptor 1 in human endothelial cells and its significant up-regulation by pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). Thus, the altered expression of chemerin and its receptors during an inflammatory process might cause dysregulated angiogenesis, leading to the development of cardiovascular disease.

However, there have been very few clinical studies that examined the influence of circulating chemerin on the atherosclerotic process. Lehrke *et al*[67] showed that circulating chemerin was positively correlated with the atherosclerotic plaque burden, as assessed by multi-slice computed tomography angiography, but that the association was lost after adjusting for established cardiovascular risk factors. Very recently, we showed that the circulating chemerin level was an independent risk factor for arterial stiffness even after adjusting other cardiovascular risk factors[68].

CONCLUSION

Various adipokines have been reported to directly modulate the atherogenic environment of the vessel wall by regulating the function of endothelial, arterial smooth muscle, and macrophage cells. Therefore, the identification of a novel adipokine that regulates the atherosclerotic process might provide new opportunities for developing more effective approaches for preventing cardiovascular disease.

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P- Reviewers: Cardoso CRL, Papazoglou D, Tomkin GH
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Hepatocyte growth factor, a biomarker of macroangiopathy in diabetes mellitus

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Received: December 27, 2013 Revised: March 1, 2014
Accepted: May 31, 2014
Published online: October 15, 2014

Abstract

Atherosclerotic involvements are an essential causal element of prospect in diabetes mellitus (DM), with carotid atherosclerosis (CA) being a common risk-factor for prospective crisis of coronary artery diseases (CAD) and/or cerebral infarction (CI) in DM subjects. From another point of view, several reports have supplied augmenting proof that hepatocyte growth factor (HGF) has a physiopathological part in DM involvements. HGF has been a mesenchymal-derived polyphenic factor which modulates development, motion, and morphosis of diverse cells, and has been regarded as a humor intermediator of epithelial-mesenchymal interplays. The serum concentrations of HGF have been elevated in subjects with CAD and CI, especially during the acute phase of both disturbances. In our study with 89 type 2 DM patients, the association between serum concentrations of HGF and risk-factors for macrovascular complications inclusive of CA were examined. The average of serum HGF levels in the subjects was more elevated than the reference interval. The serum HGF concentrations associated positively with both intimal-media thickness (IMT) (r = 0.24, P = 0.0248) and plaque score (r = 0.27, P = 0.0126), indicating a relationship between the elevated HGF concentrations and advancement of CA involvements. Multivariate statistical analysis accentuated that serum concentrations of HGF would be associated independently with IMT (standardized = 0.28, P = 0.0499). The review indicates what is presently known regarding serum HGF might be a new and meaningful biomarker of macroangiopathy in DM subjects.

Key words: Hepatocyte growth factor; Diabetes mellitus; Carotid atherosclerosis; Macroangiopathy; Biomarker

Core tip: Hepatocyte growth factor (HGF) has been a mesenchymal-derived polyphenic factor which modulates development, motion, and morphosis of diverse cells, and has been regarded as a humor intermediator of epithelial-mesenchymal interplays. The serum levels of HGF in diabetes mellitus (DM) subjects might be assayed by balancing of stimulators (hypertension, atheromatous arteriosclerosis, *etc*.) and suppressors (hyperglycemia, transforming growth factor-, angiotensin Ⅱ, *etc*.). The elevated serum level of HGF might have been regarded as an indicator of the DM involvements seriousness. Accordingly, the concentration of serum HGF might be a new and meaningful biomarker of macroangiopathy in DM subjects.

Original sources: Konya H, Miuchi M, Satani K, Matsutani S, Tsunoda T, Yano Y, Katsuno T, Hamaguchi T, Miyagawa JI,

Namba M. Hepatocyte growth factor, a biomarker of macroangiopathy in diabetes mellitus. *World J Diabetes* 2014; 5(5): 678-688 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/678.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.678

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disturbance and one of the principal chronic diseases internationally. The planetary number of diabetic (DM) patients is approximated at 382 million (mill) in 2013, and it is anticipated to be over 592 mill by the year 2035[1]. Close to 5.1 mill the dead in the 20-79 years aged group might be due to DM in 2013, elucidating 8.4% of the global all-cause deathrate[2]. In addition to the effect on the subjects' life quality, the microvascular [diabetic retinopathy (DR), diabetic nephropathy (DN), neuropathy] and macrovascular complicating diseases (coronary heart diseases, peripheral artery diseases, and stroke) of DM also increase the internal healthcare spendings. Approximated planetary healthcare expendings to care and preclude DM and its complicating diseases are anticipated to total least-wise 548 billion USD in 2013. By 2035, this number is proposed to surpass some 627 billion USD[3]. Worldwide, DM is probable to be the fifth leading killer[4].

DM individuals, both type 1 DM (T1DM) likewise T2DM, have an elevated hazard of growing endorgan dysfunction. In a clinical manner, the conception of DM cardiac myopathy is determined as cardiac ventricle damage that arises irrespective of hypertension (HTN) and coronary artery disease (CAD), namely as a discrete primitive disorder course that generates secondarily to a damage of metabolism and leads to morphological and functioning anomalies of the myocardia guiding to heart failure (HF). Human DM cardiac myopathy has been chiefly demonstrated by the damage of diastole, that might introduce the the damage of systole growing[5]. Intriguingly, solely roughly 30% of T2DM and T1DM subjects make grow DN, in contradistinction to DM cardiac myopathy that is existed in half of T2DM subjects and DR investigated in over 90% of T1DM individuals[6,7]. It suggests a distinct timecourse of DM endorgan disorder. Therefore, in a differential manner, respective cell types would be exact to hyperglycemia-caused disturbance possibly for sake of distinct expression or activeness of molecular factors would be in charge of damage activating and progression[8].

Atherosclerotic complicating diseases are an essential causal element of prognosis in T2DM, with carotid atherosclerosis (CA) being a common risk-factor for prospective crisis of CAD and/or cerebral infarction (CI)[9,10]. Some molecules, such as high-sensitivity C-reactive protein (hs-CRP) and interleukin-18, would have been presented to be atherosclerotic biomarkers[11,12]. Preclusion of DM and its involvements, early invention of disease stages, and interventions that would act in the presence of hyperglycemia to avoid, retard or inverse the involvements are the principal concerns. Biomarkers have been investigated for understanding the structures of the evolution and progress of DM involvements[13]. This review presents what is currently known regarding serum hepatocyte growth factor (HGF) level might be a new and meaningful biomarker of DM macroangiopathy.

PLEIOTROPHIC EFFECTS OF HGF

HGF has been a mesenchymal-derived polyphenic factor which modulates diverse cells development, motion, and morphosis and it is thought that HGF would be a body fluid intermediator of epithelial-mesenchymal interplays. HGF has been distinguished as a new element of the family of endothelium-specific growth factors and a topical HGF system, configured HGF and its particular receptor mesenchymal epithelial transition factor (c-MET, MET), would have been presented in blood vessel cells both *in vivo* and *in vitro*[14-17]. Additionally, there is the proof that HGF induces the security and/or restoration of vascular endothelical cells hurt by HTN, with elevated serum HGF concentrations happening dependent on endothelial cell dysfunction[18,19]. HGF has been a polyphenic cytokine related to tissue security and restoration of the vascular endothelia[13-18]. Furthermore, it has been demonstrated that HGF would have in vitro mitogenic action in cultivation systems, and is deemed to be a new angiogenetic growth factor[20] (Figure 1). Some of investigations have demonstrated that HGF/scatter factor (SF) is represented by smooth muscle cells (SMCs) but works on vascular endothelical cells, not SMCs in the artery wall[17]. Nevertheless, different investigations have suggested that SMCs can react to HGF/SF[15,16]. McKinnon *et al*[21] have restudied expression and action of HGF/SF and its receptor MET in artery SMC and vascular endothelical cell cultivations and in total arteries after superficial or deep damage or atherogenicity. High-density cultivations of SMCs brought about HGF/SF but did not express MET, meanwhile SMCs, at the leading-edge of damaged cultivations, expressed both ligand and receptor and displayed a conspicuous motion and development reaction to HGF/SF. In accordance with these outcomes, HGF/SF and MET expression was indiscernible in the media of undamaged carotid arteries but was caused after deep artery damage in areas of SMC migration in the neointima. In addition, strong MET expression was found in the SMCs of the atheromatous arteriosclerotic focuses of homozygous apoE(-/-) mice, meanwhile HGF/SF was expressed by macrophage-derived foam cells. These results showed that MET was caused in migrating and proliferating SMCs and that HGF/SF and MET were key agents of the SMC reaction in atherogenicity[21].

ANTI-APOPTOTIC ACTION OF HGF IN ENDOTHELIAL CELLS

It was focalized that the character of HGF would be a new, element of the angiogenetic proliferators[15,18].

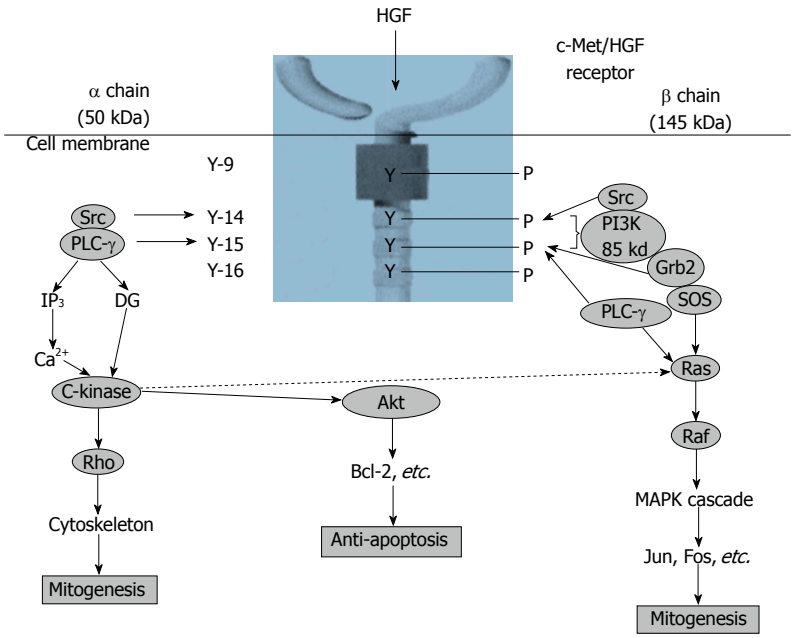

Figure 1 Signal transduction system of hepatocyte growth factor[20]. HGF: Hepatocyte growth factor; PI3K: Phosphatidylinositol 3-kinase; PLC-γ: Phospholipase C-γ; Grb2: Growth factor receptor-bound protein 2; SOS: Son of sevenless.

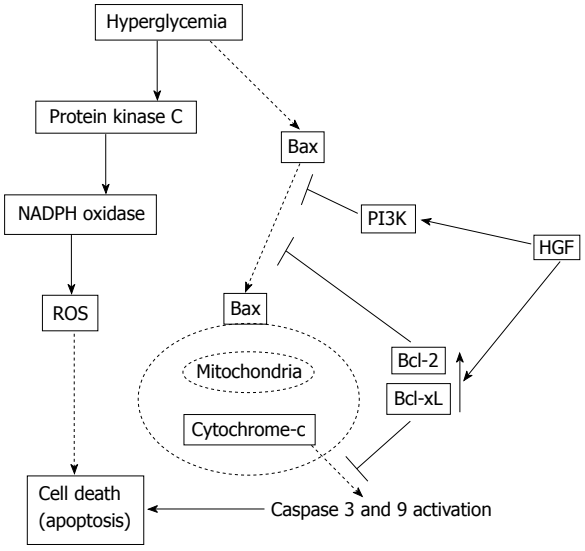

Figure 2 Potential mechanisms of anti-apoptotic effect of hepatocyte growth factor[33]. HGF: Hepatocyte growth factor; PI3K: Phosphatidylinositol 3-kinase; ROS: Reactive oxygen species.

Regional vascular HGF output was reduced by elevated glucose *via* the transforming growth factor-β (TGF-β) activating[22]. It was crucial that genetically modified HGF elevated bcl-2 protein without impacting bax protein and weakened the elevated glucose-caused caspase 3 and 9 activating[23]. The anti-apoptotic effect of HGF by bcl-2 initiation was possibly efficient against not merely elevated glucose conditions, but other stimulus related to activating of the mitochondrial-mediated apoptotic pathway, because HGF weakened caspase 3 activating stimulated by tumor necrosis factor-alpha by the phosphatidylinositol 3-kinase pathway, that was related to Akt activating[24]. These anti-apoptotic effects of HGF are not only unequaled as vascular endothelial growth factor (VEGF) and fibroblast growth factor but also demonstrated such effects. In addition, expression of VEGF and its receptor were reduced in the DM rats myocardia[25], as well as HGF[26]. Nonetheless, an unequalled latent mode of HGF is the capacity of immediate relationship between bcl-2 and MET due to bag-1 protein. The bag-1 protein has been accounted to interplay with the bcl-2 protein and to collaborate with the bcl-2 protein to inhibit apoptosis[27]. Of consequence, the bag-1 protein seems to reduce apoptosis by binding to bcl-2, the raf-1 protein kinase, and MET[28]. Besides, the conjunctive activating of these bcl-2-related genes might take part the apoptosis inhibition by HGF. It has been shown that bcl-2 affects antiapoptotic action by two modes: segregation of the executes of two major caspases-pro-caspase 9 and pro-caspase 8-and suppression of apoptogenic mitochondrial alterations, inclusive of cytochrome c secrete and loss, leading to apoptosis inductive factor secrete from isolated mitochondria[29,30]. In addition, it has been described that HGF could prevent against cell death by the phosphorylation of bad *via* phosphatidylinositol 3-kinase and augment bcl-xL[31], and bax translocation can be modulated by a configurational alteration leading to the exposition of its BH3 domain, and phosphatidylinositol 3-kinase precludes apoptosis by the depression of configurational alteration of the bax BH3 epitope[32]. These findings suggested that vascular endothelical cell death, particularly apoptosis, in hyperglycemia could be weakened by addition of growth factors, which would be potent anti-apoptotic factors (Figure 2)[33].

SERUM HGF CONCENTRATION IN T2DM PATIENTS

The previous studies showed that hyperglycemia reduced

regional HGF output in blood vessel unstriped muscle cells and vascular endothelical cells[22,34], Morishita *et al*[22] postulated that hyperglycemia influences HGF output in diverse apparatuses, such as the renal. If so, the serum level of HGF might be suppressed in DM. In a KKAy mice model of T2DM, the concentration of serum HGF was conspicuously decreased as compared to that in 14 wk of aged control mice[35], while renal and cardiac HGF concentration were remarkably decreased in KKAy mice as compared to those in C57BL mice. In this way, they moreover evaluated their hypothesis in human subjects in order to explore the association between the level of serum HGF and the severeness of T2DM. As supposed, the concentration of serum HGF was remarkably inversely correlated with HbA1c level[35]. In an interesting manner, the concentration of serum HGF in T2DM subjects was remarkably lower than that in non-DM subjects. There was no meaningful divergence in the serum HGF concentration between male and female subjects in either group. It is remarkable that there is a divergence between increased serum HGF in hypertensive (HTN) and decreased serum HGF in T2DM, whereas the tissue HGF levels are decreased in both diseases. The liver, lung and kidney are supposed to be major sources of serum HGF. High blood pressure (BP) in HTN patients does not cause injury to the liver or lung, while high blood glucose is known to influence the liver of such patients. Indeed, activation of serum TGF-β, a strong negative regulator of HGF, has been shown to be increased in T2DM patients[36]. In HTN, on the other hand, because the liver and lung are not injured by high BP, they can secrete HGF into serum in response to HTN damage. It is likely that this difference in the changes of serum HGF level between HTN and T2DM is due to the different influences exerted by high BP and high blood glucose on the major source of circulating HGF. In a contrasting manner, the concentration of serum HGF in T2DM subjects with HTN was markedly more elevated than that in the normal control subjects or that in T2DM subjects with no HTN.

Additionally, the concentration of serum HGF in all T2DM subjects was conspicuously correlated with systolic, but not with diastolic, BP. The concentration of serum HGF in T2DM subjects without HTN complications was markedly more elevated than that in the normal control subjects. The concentration of serum HGF in T2DM subjects with HTN involvements was higher than that in the other subjects. Nishimura *et al*[37] examined the association between the level of serum HGF and proliferative DM retinopathy (PDR), which is characterized by the major characteristic of retinal neovascularization. They found that the serum HGF concentration in T2DM individuals with no DR was more reduced than that in non DM individuals. Serum HGF concentration was elevated in PDR subjects who had not received photocoagulation, but not in those who had received photocoagulation. They concluded that the measurement of serum HGF may be helpful in predicting the presence of PDR in T2DM subjects. Afterwards, they reported that individuals with advanced grades of arteriosclerotic changes had higher serum HGF levels[38]. By contrast, they did not show a positive relationship between HTN and the level of serum HGF. As they included patients treated with antihypertensive drugs, it would be useful to assess the correlation between the level of serum HGF and BP of patients not treated with such drugs. It has also been reported that serum HGF was increased within 3 h after the beginning of pectoralgia in acute myocardial infarction (MI) subjects[39]. Attractively, increased HGF concentrations were conspicuously more common than those of creatine kinase (CK) within 3 h, and the increased level associated well with that of serum CK at 6-9 h after the beginning of acute MI. Therefore, HGF assay is a precise early checkup approach of the presence of arteriosclerotic lesions and acute MI. Serum HGF concentration may be a beneficial biomarker for investigating the cardiovascular disease development.

HGF is a member of the kringle proteins family, distinguished by a triple disulfide loop configuration (kringles) that communicates protein/protein and protein/cell interplay[40]. Consequently, HGF might serve a function in the modulation of thrombi and atheromatous arteriosclerosis. The kringle family to which HGF belongs contains tissue-plasminogen activator (t-PA), plasminogen, apolipoprotein (a) [Lp (a)] and urokinase. The effect of other factors associated with thrombi and atheromatous arteriosclerosis on the serum concentration of HGF was also evaluated, with the outcome that there was no remarkable relationship between the serum concentrations of HGF and total cholesterol. Likewise, the levels of t-PA, plasminogen activator inhibitor 1 and Lp (a) did not demonstrate any relationship with the concentration of serum HGF.

SERUM HGF CONCENTRATION IN T1DM PATIENTS

Nowak *et al*[41] hypothesized that the high level of HGF determined in T1DM subjects might be a significant DR progression biomarker and that the concentration of HGF might be a PDR risk indicator. Average levels of serum HGF in the control subjects were remarkably lower than in the T1DM subjects. They determined a meaning increment in the concentrations of serum HGF in T1DM subjects with PDR in comparison with the control subjects. Average concentrations of serum HGF were conspicuously higher in T1DM subjects with PDR than in T1DM subjects without DR. The concentration of HGF might be elevated in T1DM subjects with PDR, and levels increment with the DR progression, indicating that HGF takes on a role in the etiology of PDR in T1DM patients[41].

HGF AND CI AND CAD

The concentrations of serum HGF are elevated in sub-

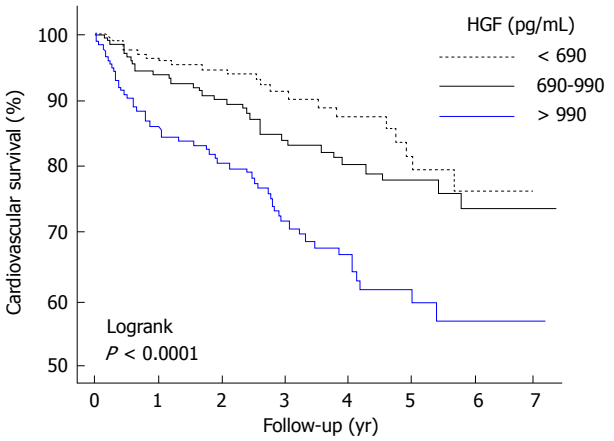

Figure 3 Kaplan-Meier survival curves in accordance with the tertiles of hepatocyte growth factor. The survival curves shows a worsened result for subjects with elevated hepatocyte growth factor (HGF) concentrations[45].

jects with CI and CAD, especially in the acute stage of both damages[39,42]. Besides, Nakamura *et al*[35] have shown that the concentrations of HGF were elevated in T2DM subjects who had HTN involvements such as arteriosclerosis. In addition, it has been described that the serum levels of HGF would be elevated in subjects during the beginning of acute MI and ischemic apoplexy[39,42].

Rajpathak *et al*[43] carried out a nested case-control study to constructively assessed the relationship between plasma HGF and ischemic apoplexy risk within the Women's Health Initiative Observational Study, a cohort of 50 to 79 years aged postmenopausal women. Base line plasma HGF concentrations were associated positively with body mass index (BMI), systolic BP, low-density lipoprotein cholesterol, insulin resistance, and inflammatory markers, such as CRP, and negatively with high-density lipoprotein cholesterol (HDL-C) (all $P < 0.05$). Base line plasma HGF concentrations were more elevated among cases than control subjects (geometric means, 601.8 *vs* 523.2 pg/mL; $P = 0.003$). Circulating plasma HGF levels are correlated with an elevated incidental ischemic apoplexy risk, extraneous to obesity and other cardiovascular disease risk-factors, amongst the 50 to 79 year aged postmenopausal women[43]. The white matter lesions (WML) existence is an essential predictive factor for the apoplexy onset. Increased levels of HGF are correlated with a high T2DM subjects death rate. The BMI was more elevated in the WML-positive subjects than that in the WML-negative subjects. Plasma concentrations of triglycerides were higher while HDL-C was more reduced in the WML-positive subjects than in the WML-negative subjects. Fasting plasma glucose ($P < 0.0001$), insulin levels ($P < 0.0001$), HOMA index ($P < 0.0001$) and HGF ($P < 0.0001$) levels were more elevated in the WML-positive subjects than in the WML-negative subjects. Multiple regression analysis showed that WML was independently prognosticated by the elevated HGF and insulin resistance ($P < 0.0001$ and $P < 0.0001$ respectively). The auxiliary investigation demonstrate that the WML existence was correlated with the increased HGF and insulin resistance in Japanese T2DM subjects[44].

Presently, the utilization of HGF as a biomarker of circulatory system disorder has been in the potent controversy as some reports showed elevated serum HGF level in HF subjects. Lamblin *et al*[45] studied the predictive value of 2 cytokines, HGF and, VEGF in subjects assessed for a decreased left ventricular ejection fraction (LVEF). Nevertheless, elevated concentrations of HGF were powerfully correlated with biomarkers of congestive HF severeness for example more elevated New York Heart Association class and more reduced LVEF, likewise clinical results inclusive of both cardiac and total deathrate (Figure 3). The relationship of HGF with harmful results continued multivariate statistical analysis that integrated latest style of risk-factors for example brain natriuretic peptide (BNP) and peak oxygen consume, a significant stage when evaluating the novel biomarker. Thoroughly, the concentrations of HGF would be more elevated in subjects with a heart trouble [1001 (741-1327) pg/mL] than in the subjects without it [773 (610-1045) pg/mL, $P < 0.000$]. Comparable outcomes would be determined when total deathrate was conceived. The concentrations of HGF would be more elevated in the subjects that deceased of any cause [940 (748-1306) pg/mL] than in subjects that would not. In an important way, the levels of HGF were intensely correlated with age, DM, and all biomarkers of congestive HF severeness. Accordingly, the survival curves suggested a worsened result for subjects with high HGF concentrations. In addition, Lamblin *et al*[46] investigated a first anterior Q-wave MI subjects. It was found that the plasma concentrations of HGF would be positively correlated with left ventricular (LV) volumes, wall motion systolic index, early transmitral velocity to mitral annular early diastolic velocity ratio, and BNP concentrations. Elevated concentrations of HGF would be correlated with more elevated CRP concentrations. Meanwhile, the concentrations of HGF were inversely correlated with LVEF. Multiple regression analysis demonstrated that both CRP and BNP were independently correlated with the concentrations of HGF at 3 and 12 mo. Subjects that deceased or were rehospitalised for HF during follow-up had more elevated concentrations of HGF at 1 mo, 3 mo, and 1 year after MI. Therefore, the circulating concentrations of HGF associated with all markers of LV remodeling after MI and would be correlated with rehospitalization for HF[46].

Susen *et al*[47] investigated the correlation between base line concentrations of the serum angiogenic growth factors, VEGF and HGF, and clinical result in 488 consecutive subjects related to elective percutaneous coronary revascularization (PCR) with no heparin pre-treatment. This primary endpoint, a complex of decease and MI, happened in 44 subjects at a median follow-up of 14.9 mo. At base line, the concentrations of HGF were in relation to CRP concentrations, DM, and late clinical unstability. HGF had a notable positive correlation ($P = 0.003$) with the primary endpoint in the univariate analysis. A same trend was found for VEGF ($P = 0.11$). The only

three variables remarkably correlated with the primary endpoint were HGF (P = 0.004), CRP (P = 0.007), and DM (P = 0.04) in the multivariate Cox model. It is demonstrated that an elevated serum HGF concentration is an independent predictive factor of clinical outcomes during follow-up and is associated with other surrogate markers of the atheromatous arteriosclerosis activeness in subjects, without heparin pre-treatment, related to PCR[47].

HGF would be a magnetic biochemical marker in congested HF subjects therefore it is augmented in the circumstance of cardiac muscle cell apoptosis and active tissue repair, whereby ascertaining patients that are at elevated hazard of harmful clinical results. Nevertheless, based off of obtainable proof, the the heart disorder pathogenesis should be assumed before utilizing HGF as a biochemical marker[48].

HGF AND DM CARDIAC MYOPATHY

The part of HGF/MET signalling in tissue of heart is chiefly attached to ischemic injury and little is recognized about its part in DM cardiac myopathy. Thus HGF brings about the vascular endothelical cells preservation or reparation and reduced serum and tissue concentrations of HGF would be referred for the advance of vascular endothelical cell injury caused by DM[49], the similar would be real for tissue of heart. Generally, elevated HGF would be supposed to be an involvements biomarker. Nevertheless, regional HGF output in blood vessel cells would be presented to be remarkably depressed by elevated D-glucose[50] that indicates reduced regional HGF generation might promote the atheromatous arteriosclerotic blood vessel alterations advance likewise cardiomyocytes damage in DM. Successively, an adaptative increment of HGF in progressed DM might promote the supposition that the levels of serum HGF are increased dependent on diverse apparatus damages.

Nakamura *et al*[49] discovered a serum level of HGF decrement in DM subjects with no HTN but an increment in subjects concerned about both DM likewise arterial HTN. In the latter group, the level of HGF successively elevated with the degree of HTN and it positively associated with systole BP in DM subjects. Furthermore, both clinical and animal experimental result indicated that the serum level of HGF was inversely associated with HbA1c in patients with no involvements, demonstrating that the damage of this vascular endothelical security in line with the DM seriousness. General HGF might affect in anagenesis as a humor intermediator, nevertheless it might be deficient to accelerate anagenesis, due to a decrement in regional HGF generation. Finally, the HGF/MET signalling would play an essential part in heart injury for example DM cardiac myopathy and precise discrimination of this part might ask for a new directions for agent exploitation and to assist better prospective DM care[8].

HGF AND THERAPEUTIC DRUG

Recently, HGF has been shown to be a downstream effector of peroxisome proliferator-activated receptor (PPAR)γ agonists[51]. Sanada *et al*[52,53] demonstrated that HGF exhibited anti-inflammatory and antioxidant effects using HGF transgenic mice. In particular, the fact that HGF has potent antifibrotic effects in both the heart and kidney through blockade of the profibrotic actions induced by angiotensin Ⅱ (Ang Ⅱ) and TGF-β1, and stimulation of degradation of fibrosis *via* matrix metalloproteinase activation is the center of interest[54-56]. In an interesting manner, amongst the accepted angiotensin receptor blockers (ARBs), irbesartan and telmisartan, so-called "metabosartans" [57], were presented to comprise a singular fraction of ARBs that can also be actuating PPARγ[58,59]. Indeed, telmisartan, reduced renal fibrosis and inflammation through the PPARγ-HGF pathway, independently of Ang Ⅱ type 1A receptor (AT1aR) blocking, in a unilateral ureteral obstruction model using AT1aR knockout (AT1aR-KO) mice[60].

Kusunoki *et al*[60] further investigated whether irbesartan has specific-organ protective effects *via* the PPARγ-HGF pathway independent of AT1aR blockade in a mouse fibrosis model, because, in large clinical trials such as the Irbesartan Microalbuminuria Type 2 Diabetes in Hypertensive Patients study and the Irbesartan Type Ⅱ Diabetic Nephropathy Trial, irbesartan demonstrated potent renoprotective effects irrespective of its hypotensive action[61,62].

"Aldosterone breakthrough" found in subjects accepting longterm care with angiotensin blocking is intensely correlated with elevated risk of LV hypertrophy, poor exercise capacity, refractory proteinuria, and decreasing glomerular filtration rate *via* the profibrotic effects of aldosterone. They used salt-sensitive HTN mediated by aldosterone and 1% NaCl infusion in AT1aR-KO mice, as this has been shown to induce severe cardiac fibrosis[63,64]. They demonstrated that irbesartan, which has not merely AT1aR- blockade actions, but PPARγ agonistic actions attended by HGF expression, suppressed organ injury by aldosterone and salt treatment[65]. Second-generation ARBs such as irbesartan, which has the double effects of AT1aR blocking and PPARγ activating, may have clinical merit for the care of HTN subjects with aldosterone breakthrough.

Calcium channel blockers are accounted to have protecting actions on the vascular endothelia *in vivo* and *in vitro*. Notably, nifedipine, amongst numerous calcium channel blockers, was demonstrated to ameliorate vascular endothelical damage in HTN subjects. Yamasaki *et al*[66] investigated the immediate actions of nifedipine on smoke-caused vascular endothelical damage, because tobacco use *per se* is a principal factor in vascular endothelical cells dysfunction, likewise HTN. They studied whether nifedipine would ameliorate endothelial action in 10 normotensive tobacco users with no atheromatous arteriosclerotic risk-factors. Nifedipine did not influence BP and cardiac rate of normotensive tobacco users. They determined forearm blood flow (FBF) by strain-gauge plethysmography after 2 and 4 wk of therapy. Alterations in vasorelaxant reaction to responsive hyperemia were

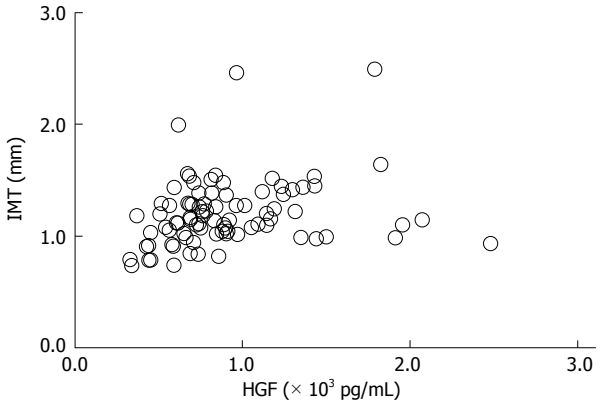

Figure 4 Relationship between serum hepatocyte growth factor and intimal-media thickness in type 2 diabetes mellitus subjects (r = 0.24, P = 0.0248)[69]. HGF: Hepatocyte growth factor.

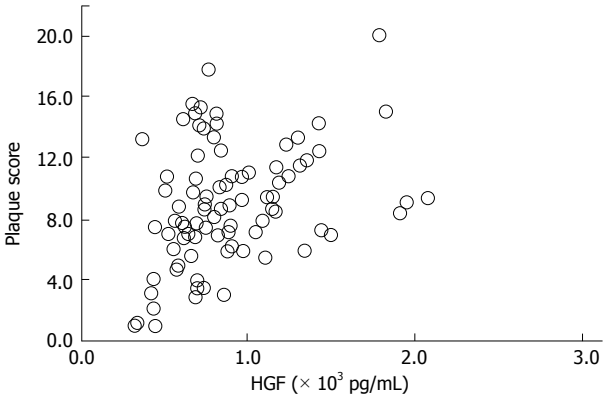

Figure 5 Relationship between serum hepatocyte growth factor and plaque score in type 2 diabetes mellitus subjects (r = 0.27, P = 0.0126)[69]. HGF: Hepatocyte growth factor.

conspicuously ameliorated in nifedipine-treated patients ($P < 0.05$), meanwhile there was no remarkable alteration in FBF reaction in controls. Furthermore, to investigate the machinery of the immediate actions of nifedipine on the endothelium, they focalized HGF, that is a new angiogenic growth factor with an antiapoptotic effect on vascular endothelical cells. Intriguingly, the serum level of HGF in tobacco users cured with nifedipine was markedly increased both at 2 and 4 wk ($P < 0.05$). Generally, these consequences indicated immediate actions of nifedipine in the endothelial damage amelioration in normotensive tobacco users. The increment in the serum level of HGF by nifedipine might bring about the vascular endothelical damage amelioration[66].

Makino *et al*[67] examined the action of calcium antagonist, benidipine, on endothelial mechanism in the essential HTN subjects, which induces endothelial damage. BP was decreased markedly. Endothelial mechanism was investigated applying FBF by strain-gauge plethysmography after 8 wk of therapy. Alterations in vasodilator reaction to responsive engorgement were notably ameliorated ($P < 0.01$), meanwhile the reaction to nitroglycerin was not altered, presenting the amelioration of endothelial mechanism. The level of serum HGF in patients cured with benidipine was grossly increased at 8 wk ($P < 0.05$). Intriguingly, an increment in the level of serum HGF by benidipine might bring about the amelioration of endothelial damage[67].

Takahashi *et al*[68] investigated whether lipid-lowering therapy (LLT) with statins would influence the leptin and angiogenic factors concentrations in CAD subjects. CAD subjects were randomised to 6 mo of intensive LLT with atorvastatin or moderate LLT with pravastatin. The plasma concentrations of leptin, Ang Ⅱ, HGF and VEGF were determined before statin treatment (baseline) and after 6 mo. Base line concentrations of leptin, Ang Ⅱ, HGF and VEGF were more elevated in the CAD subjects than in the non-CAD subjects (all $P < 0.05$). Intensive LLT reduced the concentrations of leptin, Ang Ⅱ, HGF and VEGF, while moderate LLT did not alter these concentrations. Their result displayed that LLT with atorvastatin reduces the leptin and angiogenic factors (HGF, VEGF) concentrations in CAD subjects, conceivably bringing about the favorable actions of LLT with atorvastatin in CAD[68].

HGF AND CA IN PATIENTS WITH T2DM

We conducted a clinical research to investigate the correlation between the serum HGF concentrations and the stage of CA in T2DM subjects[69]. The average level of serum HGF of T2DM patients in this clinical research was 895 +/- 408 pg/mL, a level notably more elevated than the reference values. The serum concentrations of HGF associated positively with both intimal-media thickness (IMT) ($r = 0.24$, $P = 0.0248$) and plaque score (PS) ($r = 0.27$, $P = 0.0126$) (Figures 4 and 5), indicating a correlation between the elevated HGF concentrations and development of atherosclerotic involvements.

Indeed, this was the first report presenting a notable relationship between the serum HGF concentrations and IMT and PS in T2DM subjects. Nevertheless, we failed to demonstrate a marked association between the concentrations of serum HGF and HbA1c. The clinical study outcome means the serum concentrations of HGF would be a beneficial biomarker of CA in T2DM subjects that is extraneous to entire glycemic control. Morishita *et al*[22] showed the elevated concentrations of glucose decreased the generation of HGF by vascular endothelical cells, conceivably as an outcome of apoptosis, in an in vitro study. In addition, these authors have indicated a inverse association between HGF and HbA1c in DM subjects without involvements[35]. Furthermore, the DECODE study presented that hyperglycemia after meal had an atherosclerotic action in T2DM subjects and impaired glucose tolerance subjects[70]. Collectively, these outcomes indicate additional investigations are certified to reveal the immediate or nonimmediate actions of control the level of blood glucose on both serum concentrations of HGF and the atherosclerotic involvements correlated with T2DM. It is indicated that these investigations should introduce supplemental scales like 1,5-an-

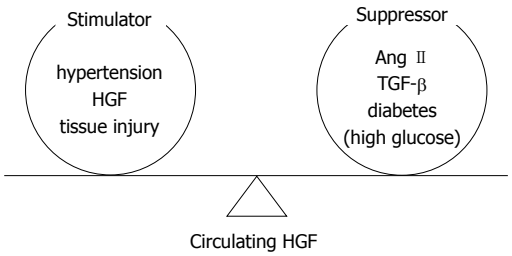

Figure 6 Determination of circulating hepatocyte growth factor level by the balance between stimulators and suppressors[19]. HGF: Hepatocyte growth factor; TGF: Transforming growth factor.

hydroglucitol and glucose level after meal. Nevertheless, using multivariate statistical analyses, we indicated that a positive relationship between the serum concentrations of HGF and IMT (standardized $\beta = 0.28$, $P = 0.0499$), we could not demonstrate any correlation between the serum concentrations of HGF and PS. The PS in the common carotid arteries (CCA) is considered as an indication of regional proliferating damages in large arteries, for instance atheromatic plaques. Since IMT and PS have discrete pathologic importance, we showed that serum HGF is a precise and characteristic biomarker for general endothelial cells proliferation. Although elevated serum concentrations of HGF would have been accounted in HTN subjects with DM[19], we could not show that the relationship was discovered between HGF and systolic BP. Contrarily, both IMT and PS associated positively with systolic BP. These outcomes would show that the concentrations of serum HGF might not be influenced by HTN intrinsically but might elevate as a secondary reaction to endothelial dysfunction that could occur during atherosclerotic progress. Hyperlipidemia, hyperglycemia and tobacco use are authenticated carotid atherosclerotic risk factors. In spite of these intense relationships, we could not show a correlation between the serum HGF concentrations and these three factors. It is potential that no correlation between serum HGF concentrations and hyperlipidemia and tobacco use was this result of the subject group not being classified in line with medical care with oral dyslipidemia therapeutic drugs, such as statin or tobacco use habit disturbance[71]. Many investigations have shown that the atherosclerotic progress in the CCA is a risk-factor for CI or MI[72,73]. IMT in those lacunar stroke subjects was not notably higher than in the no lacunar stroke subjects in our study. Contrastingly, PS in the lacunar stroke subject group was notably higher than in the no lacunar stroke group. It is demonstrated that PS is relevant to the lacunar stroke count[72], with Matsumori *et al*[42] also indicating the serum concentrations of HGF are elevated in CI subjects, especially in the preterm ischemic attack. It was discovered that both PS and IMT in ischemic heart disease (IHD) subjects would be notably more elevated than in those with no IHD. The relationship between CA and IHD has been accounted formerly[73] and moreover, it has been demonstrated that the serum levels of HGF would be elevated in acute MI subjects[39]. Our study of T2DM patients has indicated a positive relationship between the serum level of HGF and IMT and PS of the CCA. Additionally, IMT and PS would be ascertained as risk-factors for general atheromatous arteriosclerosis in both CI and CAD[69].

CONCLUSION

Actually, the serum level of HGF in DM subjects might be specified by balancing of stimulators (HTN, atheromatous arteriosclerosis, *etc*.) and suppressor (hyperglycemia, TGF-, Ang Ⅱ, *etc*.) (Figure 6)[8,19]. Accordingly, the increase of the serum HGF level might be regarded as an indicator of the DM involvements severeness. Therefore, serum concentration of HGF might be a beneficial biomarker of macroangiopathy in DM subjects.

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P- Reviewer: Gómez-Sáez J, Lysy PAG **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Cardiac autonomic neuropathy in patients with diabetes mellitus

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Received: October 15, 2013 Revised: December 2, 2013
Accepted: December 12, 2013
Published online: February 15, 2014

Abstract

Cardiac autonomic neuropathy (CAN) is an often overlooked and common complication of diabetes mellitus. CAN is associated with increased cardiovascular morbidity and mortality. The pathogenesis of CAN is complex and involves a cascade of pathways activated by hyperglycaemia resulting in neuronal ischaemia and cellular death. In addition, autoimmune and genetic factors are involved in the development of CAN. CAN might be subclinical for several years until the patient develops resting tachycardia, exercise intolerance, postural hypotension, cardiac dysfunction and diabetic cardiomyopathy. During its sub-clinical phase, heart rate variability that is influenced by the balance between parasympathetic and sympathetic tones can help in detecting CAN before the disease is symptomatic. Newer imaging techniques (such as scintigraphy) have allowed earlier detection of CAN in the pre-clinical phase and allowed better assessment of the sympathetic nervous system. One of the main difficulties in CAN research is the lack of a universally accepted definition of CAN; however, the Toronto Consensus Panel on Diabetic Neuropathy has recently issued guidance for the diagnosis and staging of CAN, and also proposed screening for CAN in patients with diabetes mellitus. A major challenge, however, is the lack of specific treatment to slow the progression or prevent the development of CAN. Lifestyle changes, improved metabolic control might prevent or slow the progression of CAN. Reversal will require combination of these treatments with new targeted therapeutic approaches. The aim of this article is to review the latest evidence regarding the epidemiology, pathogenesis, manifestations, diagnosis and treatment for CAN.

Key words: Diabetes mellitus; Cardiac; Cardiovascular; Autonomic; Neuropathy; Dysfunction; Cardiac autonomic neuropathy; Sympathetic; Parasympathetic; Heart rate variability; Spectral analysis; Diabetic cardiomyopathy; Postural hypotension

Core tip: Cardiac autonomic neuropathy (CAN) is a complication of diabetes mellitus that is often underdiagnosed but can lead to severe morbidity and mortality, due to the associated cardiovascular burden. New evidence has emerged surrounding its complex pathways, but its full pathogenesis is yet to be understood. CAN manifests in a spectrum of subclinical and clinical presentations, ranging from resting tachycardia to cardiomyopathy. Heart rate variability and scintigraphy have enabled the diagnosis at a subclinical stage, thus providing the opportunity for better prevention and treatment. However, no definite therapeutic approaches have been adopted to date, emphasizing the need for newer targeted treatments.

Original sources: Dimitropoulos G, Tahrani AA, Stevens MJ. Cardiac autonomic neuropathy in patients with diabetes mel-

litus. *World J Diabetes* 2014; 5(1): 17-39 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i1/17.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i1.17

INTRODUCTION

Diabetes mellitus (DM) is a global epidemic affecting at least 8.3% of the global population and 371 million people worldwide with a significant proportion (50%) remaining undiagnosed. It is estimated that almost one in six people are currently at risk of developing diabetes-related complications[1]. Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with diabetes and subsequently the primary goal of diabetes treatment is to reduce the burden of CVD as well as the vascular complications associated with diabetes[2,3]. Much of the CVD prevention strategies in patients with DM are based on lowering blood pressure and LDL-cholesterol levels and improving glycaemic control[4-7]. Despite that, CVD remains very common and a major cause of mortality and morbidity in patients with DM. Hence, better understanding of pathogenesis of CVD is crucial to develop new therapeutic targets.

Cardiac autonomic neuropathy (CAN) is a very common and often overlooked diabetes-related complication that has a major impact on CVD, mortality and morbidity in patients with DM[8,9]. Improving our understanding of the pathogenesis of CAN and its role in CVD, offers the potential of new treatment targets that might reduce the burden of CVD in patients with diabetes. This review aims to provide an overview of the epidemiology, pathogenesis, cardiovascular consequence, diagnosis, and treatments of CAN, with particular emphasis on the latest developments in the field.

LITERATURE SEARCH STRATEGY

We conducted a review of the original papers and review articles indexed in PubMed, Medline and Google Scholar between 1975 and 2013. We have used several terms individually or in combination including: diabetes, autonomic neuropathy, CAN, cardiovascular, cardiac, autonomic, neuropathy, dysfunction. Only articles in English and in adult population were reviewed.

DEFINITIONS AND EPIDEMIOLOGY

Based on the CAN Subcommittee of the Toronto Consensus Panel on Diabetic Neuropathy[10], CAN is defined as the impairment of cardiovascular autonomic control in patients with established DM following the exclusion of other causes. CAN, especially at the early stages, can be sub-clinical and thus as the disease progresses, it becomes clinically evident.

The prevalence of CAN varies between 1%-90% in patients with type 1 DM (T1DM) and 20%-73% in patients with T2DM (Table 1). This huge variation in CAN prevalence is due to the inconsistency in the criteria used to diagnose CAN and significant differences in the study populations, particularly in relation to CAN risk factors (such as age, gender and DM duration amongst others).

CAN has been detected at time of diagnosis of diabetes in patients with either T1DM or T2DM irrespective of age, suggesting that CAN presentation is not limited by age or type of diabetes and can occur before DM is evident clinically[11-15]. However, the duration of diabetes is an independent factor for developing CAN irrespective to diabetes type[10,16]. CAN is detected in about 7% of both T1DM and T2DM at the time of initial diagnosis[17], and it is estimated that the risk for developing CAN increases annually by approximately 6% and 2% in patients with T1DM and T2DM respectively[17-19].

Poor glycaemic control is a major risk factor for CAN progression[14,19-21]. In the Diabetes Control and Complications Trial (DCCT), intensive glycaemic control resulted in a 50% decrease in CAN incidence over the 6.5 years follow-up period[19]. This protective effect persisted 14 years after the end of the study despite the disappearance of HbA1c differences that were achieved between the groups during the randomised phase of trial[18]. Similarly, CAN has been shown to be associated with conventional CVD risk factors, such as hypertension, smoking, hyperlipidaemia and obesity[22-24]. In the Steno-2 trial of patients with T2DM and microalbuminuria, intensive pharmacological intervention targeting hypertension, hyperlipidaemia and microalbuminuria combined with behavioural treatment (exercise, diet and smoking cessation) reduced the risk of autonomic neuropathy over the course of a 7.8 years follow-up (HR = 0.37, 95%CI: 0.18-0.79)[5]. After a mean of 5.5 years following the end of the study, the same protective effect against the development of autonomic neuropathy persisted (RR = 0.53, 95%CI: 0.34-0.81, $P = 0.004$). There was also reduction in the risk for developing CVD (RR = 0.43, 95%CI: 0.19-0.94, $P = 0.04$) and overall mortality (RR = 0.54, 95%CI: 0.32-0.89, $P = 0.02$) in this study[25].

Moreover, in a large cohort of more than 1000 patients with T2DM the incidence of CAN over a 7.5 years follow-up correlated with age ($P < 0.001$) and microvascular disease ($P = 0.035$)[26]. Diabetic nephropathy (including microalbuminura), diabetic retinopathy and diabetic polyneuropathy have been widely identified as clinical predictors of CAN[23,24,27], which is not surprising as diabetic microvascular complications share common mechanisms and risk factors. The impact of gender on CAN is controversial. In a multi-centre, cross sectional study of 3250 patients with DM, CAN prevalence was no different between men and women (35% male *vs* 37% female)[28]. However, in the action to control cardiovascular risk in diabetes trial including more than 8000 patients with T2DM CAN was more prevalent in women (2.6% in men *vs* 4.7% in women for moderate severity CAN and 1.4% in men *vs* 2.2% in women for severe CAN, $P < 0.01$ for all three definitions of CAN in the study)[29].

Ethnicity has also been postulated to be a risk factor for CAN as South Asians seem to have lower rates

Table 1 Prevalence of cardiac autonomic neuropathy as reported in major studies

Ref.	Year	Country	*N* of subjects	Type of DM	Population characteristics	Diagnostic test	Criteria applied	Prevalence (%)	Comments
O'Brien *et al*[111]	1991	United Kingdom	506	IDDM	Mean age 45 yr, mean DM duration 15 yr, female 42%	HRV in response to (1) rest (2) single deep breath (3) Valsalva manoeuvre or (4) standing	At least two positive of the tests mentioned in the previous column	17	Prevalence of CAN was associated with the presence of other DM complications
Ziegler *et al*[223]	1992	Germany Austria Switzerland	130 647 524	Newly diagnosed IDDM Total IDDM Non-IDDM		CV of HRV, low- and mid-frequency bands of spectral analysis, MCR, Valsalva manoeuvre or lying-to standing	At least three positive of the tests mentioned in the previous column	7.7 25.3 34.3	
Kennedy *et al*[11]	1995	United States	290	IDDM	Listed pancreas transplantation recipients	HRV Valsalva manoeuvre		90 88	
DCCT research group[19]	1998	United States	1441	IDDM (1) primary prevention cohort (absence of end–organ damage such as retinopathy and microalbuminuria) (2) secondary intervention cohort (mild/moderate retinopathy +/- microalbuminuria)	Mean age 27 yr, female 47% duration of DM 1-5 yr (mean 2.6) primary prevention cohort 1-15 yr (mean 8.8) secondary intervention cohort	HRV Valsalva manoeuvre Postural BP	R-R variation < 15 Valsalva ratio < 1.5 Diastolic BP drop > 10 mmHg	1.6-6.2 5.5-6.3 0	These figures represent baseline characteristics
Kempler *et al*[28] (EURODIAB IDDM)	2002	16 European countries	3250	T1DM	Mean age 32 yr, mean DM duration 14 yr, female 49%	(1) R-R response to standing (2) Postural BP	R-R ratio < 1.04 or drop > 20 mmHg in systolic BP	36	Correlation with age, DM duration and HbA1c
Gaede *et al*[5,224] (the Steno type 2 study)	2003	Denmark	160	T2DM	Mean age 55 yr, female 27%, HbA1C 8.8% at baseline	(1) R-R response to breathing (2)Postural BP	R-R variation < 6 or drop > 25 mmHg in systolic BP	27.5	This figure represents baseline findings
Valensi *et al*[27]	2003	France	245 151	T1DM T2DM	Mean age 39.6 yr, mean DM duration 8.6 yr, female 43%	R-R response to (1) deep breathing (2) Valsalva and (3) standing	Criteria for abnormal tests were based on Armstrong *et al*[225] At least two positive tests (classed as moderate CAN)	21.2 20.7 33.5 20	Rate of moderate/severe CAN was higher in T1DM (18.2% and 4.8%) than in T2DM (12.3% and 2.3%) ($P = 0.031$)
Low *et al*[23]	2004	United States	83 148	T1DM T2DM	Mean age 59 yr, white 99%, female 48%	(1) Sudomotor axon-reflex test (2) Valsalva manoeuvre (3) BP and HR response to standing (4) R-R response to deep breathing	CASS $\geqslant$ 1 in two domains or $\geqslant$ 2 in one domain (sudomotor, cardiovagal, adrenergic)	54 73	This study focuses on DAN but encompasses several cardiac autonomic tests
Pop-Busui *et al*[18] (DCCT/EDIC study)	2009	United States	620 591	IDDM-former intensive Tx group IDDM-former conventional Tx group	Mean age 47 yr in both groups, mean DM duration 26 yr, female 49% and 46% respectively	R-R response to (1) deep breathing (2) Valsalva manoeuvre (3) postural BP	R-R < 15 or R-R 15-19.9 and Valsalva ratio < 1.5 or drop > 15 mmHg in diastolic BP	29 35	13/14 yr post closeout of DCCT

DM: Diabetes mellitus; IDDM: Insulin dependent diabetes mellitus; CV: Coefficient of variation; MCR: Mean circular resultant; HRV: Heart rate variability; BP: Blood pressure; CAN: Cardiac autonomic neuropathy; CASS: Composite Autonomic Severity Score; DCCT: Diabetes Control and Complications Trial; T1DM: Type 1 diabetes mellitus.

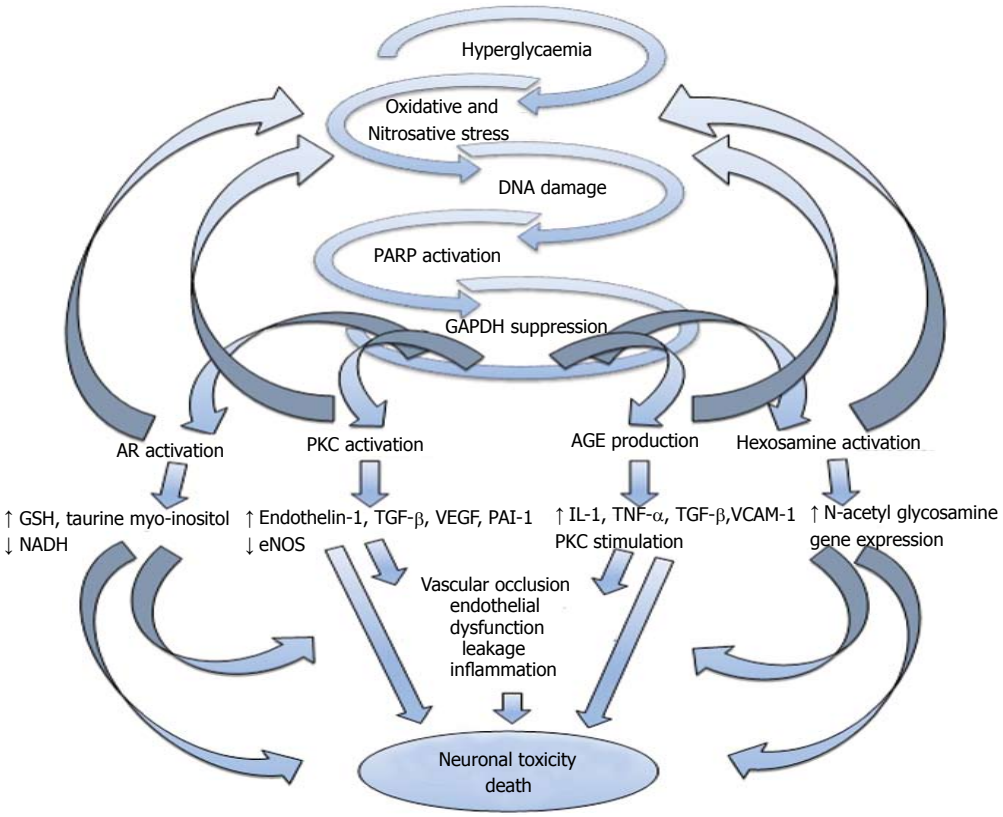

Figure 1 Summary of the mechanisms that relate hyperglycaemia to microvascular complications in patients with diabetes. PKC: Protein kinase C; AGE: Advanced glycation end-products; PARP: Poly ADP-ribose polymerase; GAPDH: Glyceraldehyde-3 phosphate dehydrogenase; GSH: Glutathione; NADH: Nicotinamide adenine dinucleotide; TGF-β: Transforming growth factor; VEGF: Vascular endothelial growth factor; PAI-1: Plasminogen activator inhibitor-1; eNOS: Endothelial nitric oxide synthase; IL-1: Interleukin 1; TNF-α: Tumour necrosis factor-α; VCAM-1: Vascular cell adhesion molecule 1.

of peripheral neuropathy than White Europeans with DM[30]. More specifically, the prevalence of small fibre neuropathy was significantly lower in Indian Asians than in Europeans (32% *vs* 43% respectively, $P = 0.03$) and mean nerve conduction velocity Z scores (measuring large fibre neuropathy) were superior in Asians compared to Europeans (mean ± SD 0.07 ± -0.62 *vs* -0.11 ± 0.60, $P = 0.007$). However, using heart rate variability (HRV) spectral analysis as well as frequency and time domain analysis showed no difference in CAN prevalence between South Asians and white Europeans (Tahrani *et al*, unpublished data).

PATHOGENESIS OF CAN

The exact pathogenesis of CAN is complex and remains unclear. Most of the proposed mechanisms of neuronal injury are based on models of somatic rather than autonomic neuropathy. Although many of these mechanisms might be shared between autonomic and somatic neuropathies, differences do exist as shown by the Steno-2 trial (described above) in which the multi-factorial intervention (including intensive metabolic control and lifestyle changes) slowed down the progression of autonomic but not somatic neuropathy.

Hyperglycaemia induced neuronal injury and ischaemia

The pathogenesis of CAN is likely to be multi-factorial[31] and to involve several mechanisms and pathways that lead to neuronal ischaemia or direct neuronal death/dysfunction (Figure 1). Hyperglycaemia and the adverse metabolic environment in patients with DM result in increased oxidative and nitrosative stress[17], which can cause direct neuronal damage/dysfunction as well as endothelial dysfunction resulting in neuronal ischaemia. Neuronal axons are rich in mitochondria which makes them particularly susceptible to the direct and indirect effects on oxidative and nitrosative stress[32].

Increased oxidative stress results in poly ADP-ribose polymerase activation which when coupled with other activated downstream pathways including the polyol pathway, advanced glycation endproducts production, protein kinase C and the hexosamine pathway are thought to contribute to glucose toxicity[33-36]. These different pathways can in return exacerbate oxidative stress and can induce changes in gene expression, transcription factors, diverse cellular products disrupting several cellular functions and the communication between the cell and the surrounding matrix all of which leads to neuronal dysfunction and death[37-39]. These pathways also result in impaired microvascular-- regulation and endothelial dysfunction by different mechanisms, including increase in plasminogen activator inhibitor-1 and endothelin-1 production and impairment of endothelial nitric oxide (NO) synthase and NO actions[40,41]. This can lead to reduction of neurovascular perfusion, dysfunction and cellular apoptosis[42].

Autoimmunity

The role of autoimmunity has also been explored particularly in patients with T1DM. The presence of complement-fixing antibodies against sympathetic and parasympathetic tissues in patients with insulin-dependent diabetes and their correlation with CAN was described in the early 90s[43,44]. In a study of 78 patients with DM,

the prevalence of phospholipid autoantibodies (PLA) in the patient's serum was significantly higher in those tested positive for autonomic neuropathy (88% of the patients with autonomic neuropathy *vs* 32% of those without, $P < 0.001$) and there was a strong correlation between the PLA titre and total neuropathy score ($r^2 = 0.58$, $P = 0.0002$)[45]. Granberg *et al*[46] demonstrated in a group of patients with T1DM that patients positive for complement-fixing antibodies to the sympathetic ganglion, vagus nerve and adrenal medulla had a significant higher risk to develop cardiac autonomic dysfunction (measured by the E/I ratio during deep inspiration and HRV to postural change) over a 6-year follow-up (RR = 7.5, 95%CI: 1.72-32.80). There are, however, conflicting reports whether these auto-antibodies contribute to the pathogenesis of autonomic neuropathy or represent rather incidental findings and can be attributed to autoimmunity against concurrent conditions, such as thyroid disease[47]. A recent study of mixed T1DM and T2DM patients concluded that neither peripheral nor CAN was associated with the presence or the levels of Neuropeptide Y Autoantibodies[48].

Residual β-cell function

Several studies have shown a protective effect of residual β-cell function (*i.e.,* C-peptide levels) on the development and incidence of microvascular complications (including CAN) in patients with T1DM[49,50]. The exact mechanisms for these associations are not clear but it is thought that the C-peptide activates Na/K channels, lowers inflammation and improves NO bioavailability and endothelial function[51,52]. Small RCTs have shown beneficial effect of C-peptide treatment on CAN parameters[53].

Genetic factors

More recently data suggesting genetic predisposition to CAN have emerged. In a study of 154 patients with T2DM, TCF7L2 gene was found to be strongly associated with the presence of CAN, as assessed by deep breathing, lying to standing, Valsalva manoeuvre and postural hypotension tests (OR = 8.28, $P = 0.022$ for the rs7903146 allele)[54]. Another study on healthy Japanese individuals showed that the T393C polymorphism of the gene encoding the Gs-protein-α-subunit (GNAS1) is significantly associated with cardiovascular autonomic dysfunction, detected with power spectral analysis ($P < 0.05$ for TT + TC *vs* CC polymorphism)[55]. Twins studies, however, failed to show an association between CAN and genetic factors[56].

Obstructive sleep apnoea

Obstructive sleep apnoea (OSA) is emerging as another possible factor in the development of CAN. OSA is very common in patients with diabetes and has been associated with increased sympathetic tone in patients without diabetes[57,58]. The interrelationship between OSA and CAN in patients with DM requires further investigation and is likely to be bidirectional. While the intermittent hypoxia that occurs in OSA could lead to increased oxidative stress, nitrosative stress, and impaired microvascular complications which could lead to CAN[59], CAN on the other hand could lead to changes in upper airways tone and changes in respiratory drive which could predispose the patient to OSA. One recent study presented in the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes 2012 meeting showed that the prevalence of CAN was similar in patients with T2DM with and without OSA, but CAN severity was worse in the OSA group (Tahrani *et al*[59], unpublished data). Furthermore, the presence of CAN was associated with more severe apnoea/hypopnea episodes (Tahrani *et al*[59], unpublished data).

NATURAL HISTORY OF CAN

DM affects the autonomic (as well as the peripheral) nervous system in an ascending length-dependent manner. The vagus nerve, which anatomically is the longest autonomic nerve and physiologically mediates 75% of the overall parasympathetic activity, tends to be involved early in the course of CAN development. The early stages of CAN therefore involve reduction in parasympathetic activity, which results in sympathetic predominance. This increase in sympathetic tone continues until the latest stage of CAN when sympathetic denervation ensues, which spreads gradually from the apex to the base of the heart[60,61].

CAN is divided into a sub-clinical and a clinical stage. During the initial sub-clinical stage, CAN is detected through abnormalities in frequency and time domains of the spectral analysis of HRV and the Baroreflex Sensitivity (BRS) tests, as well as an increased torsion of the left ventricle (LV) on cardiac imaging before the development of abnormalities in standard cardiac autonomic reflex testing (CART) (please see below for details)[62-67]. Studies have shown that these abnormalities can even be present at the time of diagnosis of DM[63]. CAN progresses and parasympathetic denervation is followed by compensatory sympathetic overdrive, resulting in abnormal CARTs followed by symptomatic CAN in which the clinical manifestations become apparent (please see below). At the stage of sympathetic denervation, autonomic dysfunction correlates clinically with postural hypotension[63] (Figure 2). The time scale for the progression of subclinical CAN to the development of abnormal CART is unclear; similarly the natural history of the development of early cardiac abnormalities (such as torsion or deficits in myocardial perfusion or cardiac energetic) and its relationship to subclinical CAN is also unclear. But we estimate that many patients with sub-clinical CAN will develop abnormal CART and early features of cardiac involvement within 5 years of developing abnormal frequency and time domain parameters.

CLINICAL MANIFESTATIONS OF CAN

Resting tachycardia

Resting tachycardia is a common manifestation of CAN

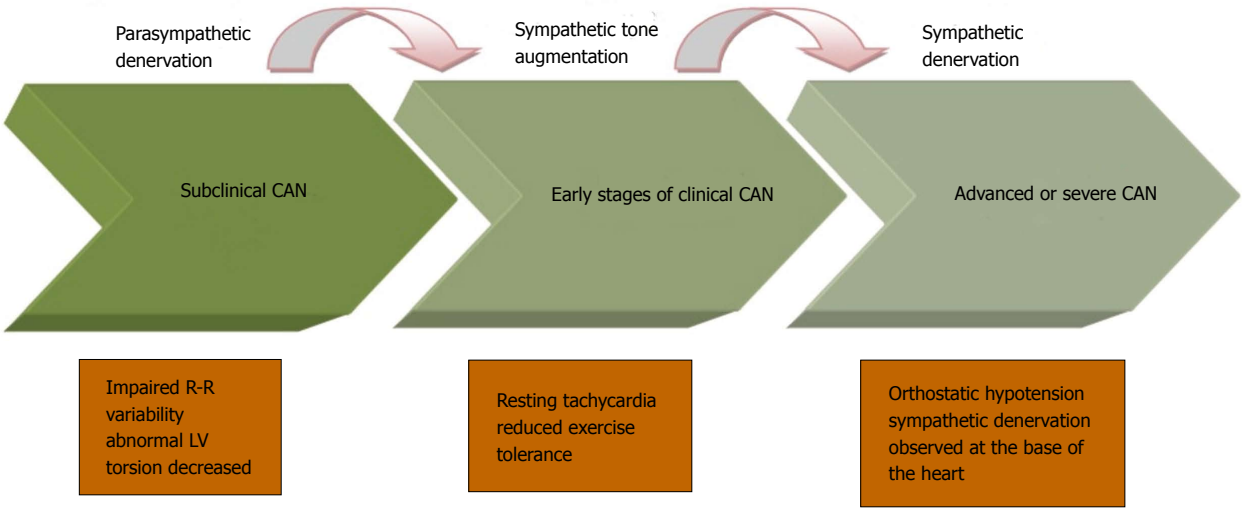

Figure 2 Natural progression of CAN and correlation with clinical signs and symptoms. CAN: Cardiac autonomic neuropathy; LV: Left ventricle.

that occurs at a relatively early stage of the disease. A HR of 90-130 beats per minute (bpm) can be observed and is associated with a reduction in parasympathetic tone followed by increased sympathetic activity as CAN progresses[68]. A fixed HR which does not change during sleep, exercise or stress is a sign of complete cardiac denervation[69]. Moreover, poor HR response to adenosine is associated with higher risk for adverse cardiac events[70], including all-cause and CVD mortality[71]. Hence, resting HR can be used as a diagnostic and prognostic tool in patients with DM after excluding other causes of tachycardia[10].

Exercise intolerance

Impaired blood pressure, HR and cardiac stroke volume in response to exercise in the absence of structural or coronary cardiac disease are all features of CAN[69]. As disease progresses, the parasympathetic-sympathetic imbalance can lead to further impairment of the above parameters[68] which limits the diagnostic utility of exercise tolerance testing in these patients due to increased false-negative outcomes caused by blunted HR response[72]. In addition, patients with CAN should be tested using stress cardiac imaging (usually echocardiography) prior to starting an exercise program, especially those with high-risk profile[69].

Orthostatic hypotension

Orthostatic hypotension is a manifestation of advanced CAN. Orthostatic hypotension is defined as the reduction in systolic blood pressure by > 20 mmHg or in diastolic blood pressure by > 10 mmHg 2 min following postural change from supine to standing[17,19,69]. Orthostatic hypotension occurs as a result of the impairment of the sympathetic response to postural change secondary to poor norepinephrine response and abnormalities in the baro-receptor sensitivity, resulting in inadequate HR response and peripheral vasoconstriction[23,69]. Orthostatic hypotension can be aggravated by many medications that are commonly used in patients with DM such as diuretics, vasodilators, tricyclic antidepressants and insulin[63]. Similar to resting tachycardia, assessing the presence of orthostatic hypotension is of prognostic value as a marker of advanced CAN[10]. In the middle-aged general population, orthostatic hypotension has been shown to be an independent prognostic factor for CVD and all-cause mortality[73].

Silent ischaemia

CAN is associated with a prolonged subjective angina threshold (which is defined as the time between the observation of 1 mm ST depression on the electrocardiogram and the development of symptoms of angina pectoris); thus rendering patients with CAN susceptible for experiencing silent myocardial ischaemia and potentially infarction, despite being asymptomatic[74]. A meta-analysis of 12 cross-sectional studies showed that CAN is associated with silent ischaemia in patients with DM (the Mantel-Haenszel estimate for prevalence rate risk was 1.96, 95%CI: 1.53-2.51)[17]. A study of 120 patients with DM and no previous CVD found evidence that CAN (detected using the Valsalva manoeuvre, the deep breath test and lying-to-standing HRV) was a better predictor of major cardiac events [*i.e.,* myocardial infarction or myocardial infarction (MI)] than the presence of silent ischaemia (OR = 4.16, 95%CI: 1.01-17.19) but when CAN was combined with silent ischaemia the risk was even higher (5 out of 10 had a major event)[75]. A study from Spain that included 217 patients with T1DM and T2DM, found that the presence of autonomic neuropathy is independently associated with increased risk for developing silent ischaemia (as demonstrated by positive exercise test) (OR = 6.5, 95%CI: 1.3-7.9) especially when combined with other cardiovascular risk factors such as microalbuminuria[76]. In the Detection of Ischaemia in Asymptomatic Diabetic subjects study which included 1123 patients with T2DM, CAN (defined as abnormal Valsalva manoeuvre) was also a predictor of silent ischaemia (defined using stress cardiac perfusion imaging) (OR = 5.6, 95%CI: 2.6-12.4, P = 0.0001)[77].

It is evident that patients with DM and CAN are at high risk of sustaining a major cardiovascular event during exercise, due to the limited perception of ischaemic pain which could delay the appropriate and timely response to ischaemia. A recent statement from the Toronto Consensus Panel on Diabetic Neuropathy has emphasised the importance of integration of cardiac autonomic function testing into the current risk stratification pathways for patients with DM and established CVD risk factors[10].

The mechanisms underpinning relationship between CAN and silent ischaemia are not clear. Several mechanisms have been proposed including altered pain threshold, impaired afferent myocardial autonomic pathways and ischaemic processes not detected by routine electrocardiography. There has also been debate over whether the relationship between them is indeed a causative one, or both CAN and silent ischaemia are a product of coronary artery disease observed in diabetes[78,79].

Diabetic cardiomyopathy and LV dysfunction

Diabetic cardiomyopathy is a clinical entity that is characterised by changes in the biochemical signalling in the presence of a sympathetic-vagal imbalance resulting ultimately in left ventricular hypertrophy and remodelling, and therefore cardiac dysfunction in patients with DM in the absence of coronary artery disease[63]. Diabetic cardiomyopathy results in variable degrees of systolic and predominantly diastolic dysfunction in the absence of structural or valvular cardiac disease, coronary vessel disease, or hypertension[80,81]. Changes in the diastolic and/or systolic function can be identified on various diagnostic imaging modalities in otherwise asymptomatic patients and can precede the occurrence of macrovascular diabetic complications[82]. Frequently, the only detectable abnormality in the early stages of CAN is an isolated diastolic dysfunction with a normal LV ejection fraction[83] associated with high CVD morbidity[84,85].

Conventional echocardiography studies, with or without Doppler technique, showed that CAN is associated with significant reduction in the peak diastolic filling and an increase in the atrial component of diastole[69]. The introduction of new diagnostic modalities, such as the cardiac magnetic resonance imaging has allowed even more sensitive means of diagnosing and classifying diabetic cardiomyopathy even in the early stages by examining myocardial twist, torsion and strain[86]. Torsion is a measure of the apical rotation along the long axis of the heart and is followed by a rapid untwisting, occurring during the isovolumic relaxation phase[87]. Both torsion and maximal torsion rate have been found to be increased in patients with T2DM and preserved systolic function[86]. In patients with T1DM, increased torsion appears to be independent of energetic deficits but related to microvascular perfusion deficits and correlates with changes in sympathetic denervation[88,89]. Myocardial Perfusion Reserve (another diagnostic tool used for the detection of microvascular abnormalities) has been shown to detect the early stages of CAN in asymptomatic patients and to assess CAN severity[90].

There are several proposed mechanisms for the development of diabetic cardiomyopathy. The parasympathetic denervation observed in the early stages of the disease leads to a dominant sympathetic tone[91], which promotes a cascade of intrinsic metabolic changes, including the release of high myocardial catecholamine levels and catecholamine toxicity[92,93]. This catecholamine rise has been shown to induce mitochondrial uncoupling[94,95], switching energy generation on a cardiac level from myocardial glucose to free fatty acids, which is considered an inefficient energy source[96] and therefore increases the oxygen demand[94,95]. These alterations on the cardiac biochemical and cellular level, lead ultimately to programmed cell death and fibrosis[97,98], elevated oxygen consumption relevant to the cardiac work[99,100] and finally hypertrophy and remodelling of the LV[101]. Crucial mediators in the above process are the mitochondrial reactive oxygen species[102,103], insulin resistance[104] and calcium dependent apoptosis[102,105,106].

On a macroscopic level, diastolic dysfunction in CAN is associated with delayed relaxation, impaired filling and increased stiffness of the LV[107]. The previously described sympathetic predominance is a stimulator of the rennin-angiotensin-aldosterone axis, resulting in increased HR, cardiac output and peripheral vasoconstriction[108]. Studies have shown that this alteration on the cardiac profile can lead to reduction of coronary blood perfusion and diastolic dysfunction in patients with evidence of early microangiopathy[60]. Sympathetic overdrive may also lead LV wall stress and LV hypertrophy. Pop-Busui et al have recently shown in a large cohort of the DCCT/EDIC study, that CAN is associated with a mass increase as well as a concentric remodelling of the LV, independent of other risk factors[109].

Mortality/sudden death

CAN is associated with an increased mortality risk (Table 2). This was described in longitudinal studies in the early 1990s showing a 50% increase in 5 year-mortality risk in patients with DM and autonomic neuropathy compared to those without[110-113]. In a meta-analysis of 15 studies on the basis that they included patients with DM who had baseline assessment of HRV using one or more tests described by Maser *et al*[114] showed that the pooled estimated relative mortality risk was 2.14, (95%CI: 1.83-2.51, $P < 0.0001$), for those who had CAN. CAN was also found to have the strongest association with mortality amongst other risk factors in the EURODIAB IDDM Complications Study[115].

Even in patients with high CVD risk profile such as the population of the ACCORD trial, CAN was an independent predictor of all-cause mortality (HR = 2.14, 95%CI: 1.37-3.37) as well as CVD mortality (HR = 2.62, 95%CI: 1.4-4.91) after a mean follow-up of 3.5 years[29]. Interestingly the relationship between CAN and mortality was similar regardless of treatment allocation to the intensive or standard glycaemic control groups[29].

CAN was also found to be associated with a higher

Table 2 Observed mortality in significant studies in the last two decades

Ref.	Country	*N* of subjects	Type of DM	FUp (yr)	Diagnostic test for CAN	Criteria applied	Mortality figures (expressed in HR, RR and incidence)	Comments
Veglio *et al*[226]	Italy	316	T1DM	5	(1) Resting heart rate (2) HRV during deep breathing (3) BP response to standing	⩾ 2 abnormal tests	Relative risk: 3.55 (1.4-8.9) and 2.21 (0.62-7.84, $P = 0.22$) after multivariate analysis for all-cause mortality	The mortality rates were 13% and 4% in the presence and absence of CAN respectively
Gerritsen *et al*[164] the Hoorn Study	Nether-lands	446	Non-DM	9	Seven parameters assessing HRV and BP response to: (1) 3-min breathing and (2) six deep breaths	Cut–off set as the lowest 25th percentile of non-diabetic group	Only E/I had a statistically significant association with mortality- Relative Risk: 2.25 (1.13–4.45) for all cause and 2.04 (0.74–5.65) for CVD mortality	An additional four parameters showed a tendency ($P < 0.10$) for association with acc-cause mortality: mean NN, LF power, HF power, and BRS
Chen *et al*[227]	Taiwan	159 612	T2DM T2DM	7.7	HRV response to: (1) single deep breath (2) six consecutive breaths (3) standing, (4) Valsalva manoeuvre	⩾ 3 abnormal tests	All cause mortality: 29% *vs* 12% with and without CAN respectively CVD mortality: 9% *vs* 2% in pts with and without CAN	The 8-yr survival rate for pts with abnormal CAN tests was 63.6% in males and 76.4% in females, compared with 80.9% and 93.3% for patients with normal CAN tests
Wheeler *et al*[228]	United States	843	T1DM and T2DM		HRV response to deep breathing and postural BP	Drop in BP ⩾ 30 mmHg and HRV divided into 5 quintiles	Hazard Ratio: 1.49 (1.01-2.19) for all-cause mortality and 1.08 (0.69-1.70) for CVD mortality	Of the 142 patients for whom cause of death was available, 75 deaths (49.7%) were due to CVD. The lowest quintile of HRV was associated with a 50% increase in mortality after adjusting for other risk factors
Astrup *et al*[229]	Denmark	388	T1DM (197 with macro-, 191 normo-albuminuria)	10.1	HRV to deep breathing	HRV < 10 bpm at baseline abnormal E/I	in the lowest quintile of HRV. Relative Risk for orthostatic hypotension: 0.65 (0.69-1.70) Relative risk: 4.9 (2.1-11.5, $P < 0.0001$) after adjustment for traditional CVD risk factors	During follow-up, 33 Patients died from cardiovascular causes, During follow-up 54 of 104 patients died: 41 patients (80.4%) with diabetic nephropathy and 13 patients (24.5%) with normoalbuminuria. Thirty patients (55%) died from cardio-vascular causes
Astrup *et al*[230]	Denmark	104	T2DM (51 with nephropathy, 52 with normal albuminuria)	9.2	HRV to deep breathing		Hazard Ratio: 0.92 (0.87–0.98, $P = 0.005$) for HRV (1 beat/min increase)	
Soedamah-Muthu *et al*[115] the EURODIAB PCS	16 European countries	2787	T1DM	7	HRV response to standing and postural BP	R-R ratio of < 1.04 and drop in systolic BP ⩾ 20 mmHg	Hazard Ratio: 3.61 (1.49–8.76) for CVD mortality and 2.83 (1.82–4.38) for all-cause mortality.	Autonomic neuropathy and microalbuminuria were the most important independent predictors of mortality
Lykke *et al*[231]	Denmark	391	T1DM	10	HRV and QTc		All cause mortality Hazard Ratio: 2.5 (0.9–6.8, $P = 0.071$) in pts with abnormal HRV and 2.3 (1.3-4.0, $P = 0.005$) in those with abnormal QT combined hazard ratio 6.7 (1.8-25, $P = 0.005$)	Out of 34 patients with both tests abnormal, 15 died in the 10 yr period (14 from cardiovascular causes)
Ziegler *et al*[232] MONICA/ KORA Augsburg Cohort study	Germany	1560 160	Non-DM DM	9	HRV, QTc interval and QTD	Group (1) Lowest quartile for SDNN, CV and max-min R-R intervals Group (2) QTc > 440, Group (3) QTD > 60 ms	All-cause mortality Relative Risk: 0.93 (0.65-1.34)/2.02 (1.29-3.17)/0.98 (0.60-1.60) in patients without DM and 1.74 (0.95-3.18)/3.00 (1.34-6.71)/0.42 (0.06-3.16) in patients with DM for group 1/2/3 respectively	Prolonged QTc interval was an independent predictor of mortality both in patients with and without DM, Low HRV trended towards an increased risk of mortality by 73% in patients with DM but not the population without DM
Beijers *et al*[233] the Hoorn Study	Nether-lands	376 114	Non-DM T2DM	13.6	HRV and BP response to: (1) 3-min breathing, (2) six deep breaths (3) standing	Calculated z-score for each parameter and averaged into a total CAD score	Relative risk: 2.54 (1.60–4.04) for CVD mortality and 2.11 (1.58–2.81) for all cause mortality,	CAN was associated with all-cause and CVD mortality independent to other CVD risk factors and microalbuminuria
Pop-Busui *et al*[29]	United States and Canada	8135	T2DM	3.5	HRV and QTI computed from 10-s resting electrocardiograms	CAN1: lowest quartile of SDNN and highest QTI quartile, CAN2: CAN1 and resting heart rate, CAN3: CAN1 and peripheral neuropathy	Hazard ratios: 1.55 (1.09-2.21)/2.14 (1.37-2.37)/2.07 (1.14-3.76) for all-cause and 1.94 (1.20-3.12)/2.62 (1.40-4.91)/2.95 (1.33-6.53) for CVD mortality in CAN1/CAN2/CAN3 respectively	CAN was independently associated with overall and CVD mortality after adjusting for baseline CVD, DM duration, traditional CVD risk factors and medications

FUP: Follow up; HR: Hazard ratio; RR: Relative risk; HRV: Heart rate variability; BP: Blood pressure; E/I: Expiration/inspiration; SDNN: Standard deviation of normally conducted R-R intervals; NN: Normal to normal R-R intervals; LF: Low frequency; HF: High frequency; BRS: Baroreflex sensitivity; CV: Coefficient of variation; QTD: QT dispersion; QTI: QT index; DM: Diabetes mellitus; T1DM: Type 1 diabetes mellitus; CAN: Cardiac autonomic neuropathy; CVD: Cardiovascular disease.

mortality risk in patients who had myocardial infarction[116], suggesting that screening for CAN in patients with DM who suffered a myocardial infarction can be used for risk stratification[117].

CAN is also associated with increased risk of sudden cardiac death[112,113,118]. This can be explained by the increased rate of fatal cardiac arrhythmias due to the imbalance between the sympathetic and parasympathetic autonomic function[119], as well as cardiac sympathetic denervation[67]. QT prolongation which has been associated with autonomic neuropathy in several studies[120-122], can also provide an alternative mechanism, rendering patients with CAN more susceptible to suffer life-threatening cardiac arrhythmias, including Torsades de Pointes[69]. The exact relationship between CAN and sudden cardiac death remains, however, under question. As shown by the Rochester Diabetic Neuropathy Study, sudden death cases are also related to severe coronary artery disease or LV dysfunction rather than CAN itself[123]. Nonetheless, as we discussed above, CAN seems to contribute to cardiovascular mortality even in those with established coronary artery disease.

Several mechanisms have been implicated in explaining the relationship between CAN and mortality in patients with DM. Autonomic neuropathy can lead to impaired response to hypoxic state[124], reduced hypoglycaemia awareness and prolonged hypoglycaemic episodes[111]. The observed mortality can also be attributed to a direct effect of autonomic neuropathy and its microvascular complications[125] as well as to an indirect association with end-organ complications, such as nephropathy, left ventricular hypertrophy and diastolic dysfunction[100]. In addition, the lack of the physiological nocturnal parasympathetic dominance in patients with CAN can lead to nocturnal hypertension, causing LV hypertrophy[126,127] and increasing the CVD burden[93,128].

Perioperative and intraoperative complications

Patients with CAN exhibit 2- to 3-times fold increase in perioperative morbidity (perioperative complications, impaired wound healing, impaired drug metabolism) and mortality[129,130]. Patients with CAN are more likely to require vasopressor support in the theatre setting[130]. They are also prone to experience a blood pressure and HR reduction during the induction of anaesthesia, as well as severe intraoperative hypothermia[131]. The above findings can be explained by an impairment or absence of the normal vasoconstrictive response to vasodilating anaesthesia in patients with CAN[130].

Cerebrovascular disease

Unlike the strong links between CAN and CVD, there is only limited data regarding the impact of CAN on cerebrovascular disease. In a study conducted by Töyry *et al*[132] that included 133 patients with T2DM, CAN was found to be an independent risk factor for developing stroke after 10 years of follow-up (OR = 6.7, 95%CI: 1.5-29.9 for HRV response to deep breathing and OR = 1.1, 95%CI: 1.01-1.2 for lying-standing BP). In a sub-analysis of the Appropriate Blood Pressure Control in Diabetes population, including 950 patients with T2DM over a 5-year period, CAN was significantly associated with the occurrence of stroke, independent to other risk factors[133]. The later was also confirmed by a recent study including 1458 patients with T2DM who were followed up for 7 years[134].

Diabetic nephropathy

Several authors have hypothesized that CAN is involved in the pathogenesis of diabetic nephropathy, although causation has not been proven[135]. Sympathetic overactivity has been shown to cause glomerular and tubular dysfunction in diabetic animal models *via* indirect (hypertension and angiotensin Ⅱ) and direct (vascular smooth muscles proliferation, vasoconstriction, podocytes injury) insults[136]. CAN is associated with increased CVD morbidity and mortality[63,135] and with haemodynamic changes such as lack of nocturnal BP dipping (causing increased intra-glomerular pressure resulting in albuminuria)[137] and diurnal postural falls in BP (resulting in lower intra-glomerular pressure)[138] and endothelial dysfunction in humans. In addition, CAN is associated with deficits in erythropoietin production and, as a result, erythropoietin-deficiency anaemia[137]. Subsequently, CAN patients are deprived from the direct nephroprotective action of erythropoietin and thus, anaemia becomes a strong predictor of nephropathy and progression of chronic kidney disease[68]. In streptozotocin-diabetic rats, sympathetic overactivation has been shown to be involved in the pathogenesis of diabetic nephropathy[139] and renal denervation was shown to prevent glomerular hyperfiltration[140]. Hence it is plausible that CAN is involved in the development and progression of diabetic nephropathy. Several studies examined the association between CAN and either albuminuria and/or glomerular filtration rate[141-145], but all these studies had a cross-sectional design, hence causation cannot be proven, particularly that the pathogenesis of CAN is similar to other microvascular complications including diabetic nephropathy. Longitudinal studies are scarce and limited to a small number of patients with T1DM[138,146]. Hence, data regarding the longitudinal impact of CAN on diabetic nephropathy in patients with T2DM is lacking.

Lower limb complications

CAN has been proposed as a contributing factor in the development of lower limb vascular and neurological complications. Autonomic neuropathy can cause alterations in microvascular blood flow (MBF), which predispose to changes in skin structure and quality and impairment of sweat glands' innervation resulting in dry skin and increased risk of oedema and foot deformity which increases pressure on certain areas causing ulceration[147]. It is also believed that CAN, through the sympathetic denervation of the lower limb vasculature, can induce lower extremity hyperaemia, increase inflammation and erosion into the joints/bones and therefore contribute in Charcot's neuroarthropathy. As a result, the patient with Charcot will typically present with prominent peripheral

pulses due to vasodilatation and autonomic neuropathy. Power Spectral Analysis and HRV has been employed in trials for the detection of autonomic neuropathy in patients with Charcot's disease[148]. Similarly to Charcot's arthropathy, patients with recurrent vascular neuropathic ulcers appear to share analogous cardiac autonomic dysfunction, as shown by the use of HRV, Valsalva ratio and orthostatic hypotension[149].

THE DIAGNOSIS OF CAN

CARTs

Ewing *et al*[150] proposed in early 1970s five simple non-invasive tests to measure cardiac autonomic function based on the HR and blood pressure response to certain physiological manoeuvres. These tests include: (1) the HR response to deep breathing, which assesses beat to beat HR variation (R-R variation) during paced deep breathing [expiration-to-inspiration ratio (E:I)]; (2) the HR response to standing, which is expressed as the 30:15 ratio which is the ratio of the longest R-R interval (between the 20th and 40th beat) to the shortest R-R interval (between beats 5-25) elicited by a change from horizontal to vertical position; (3) the Valsalva manoeuvre which evaluates the HR response during and after a provoked increase in the intra-thoracic and intra-abdominal pressures (the patient normally exhales for a period of 15 seconds against a fixed resistance); (4) the blood pressure response to standing, which assesses the baro-reflex mediated blood pressure change following postural change; and finally; and (5) the blood pressure response to sustained handgrip, as defined by the diastolic blood pressure increase caused by the sustained muscle contraction with the use of a handgrip dynamometer[17]. The first two tests reflect defects in the parasympathetic activity (*i.e.,* the ability of the vagal nerve to slow the HR during the procedures which increases the R-R interval and hence increases the ratios), while the last two also describe changes in the sympathetic function (*i.e.*, the ability to provide appropriate BP and HR response to the activity involved)[151,152]. The autonomic changes that occur during the Valsalva manoeuvre are complex and involve both the sympathetic and parasympathetic systems[153], although the Valsalva ratio mostly represents parasympathetic activity. For more details about the autonomic changes during Valsalva please see[17].

While the above described CARTs have been widely used since their introduction, there is no evidence on the superiority of one test over another when it comes to assessing CAN[10]. However, the HR response to deep breathing is the most commonly utilised, because of its high reproducibility and specificity[154] and its ease of use[10,155]. All the tests are considered to be valid markers of autonomic dysfunction, given that end organ failure is excluded and parameters such as concomitant illness, use of over the counter medications and lifestyle factors (exercise, smoking, exercise) are taken into consideration[156].

HRV

A reduction in HRV has been associated with the early stages of clinical CAN. In healthy individuals, the beat-to-beat variability with aspiration is predominantly affected by the direct sympathetic and parasympathetic activity[62,157], as well as various other stimuli, including certain neurohumoral factors (catecholamines, thyroid hormones), temperature changes and mechanical and ionic changes in the sinus node[158]. The efferent sympathetic and vagal stimulation is characterised by synchronised discharges, modulated by central and peripheral oscillators, with the former referring to vasomotor and respiratory centres and the later to respiratory movements and arterial pressure. These synchronous neural discharges can manifest as short and long -term oscillations in the HR[63].

The R-R intervals recorded under paced breathing are transformed to generate the time and frequency domains. Conceptually, if the faster respiratory sinus arrhythmia signal and the slower mean HR changes could each be separated from the patient's cardiogram and analyzed independently, the result would yield a measure of Vagal outflow from the respiratory sinus arrhythmia and a measure of sympathetic activity from the changes in mean HR. Effectively this is what is accomplished in the frequency- or spectral-domain. Spectral analysis of respiratory sinus arrhythmia provides the indication of where in the frequency domain the Vagus is influencing the heart. The frequency domains are generated using continuous wavelet transform method (Fourier transform) and separated to three basic components: very-low-frequency, low-frequency (LF) and high-frequency (HF)[61,159]. HF represents vagal activity, whereas LF is attributed to the combined effect of sympathetic and parasympathetic influence[62,160]. Modern software (such as ANSAR technology) adjusts for the respiratory rate, hence simplifying the process. Parameters generated include: respiratory frequency (Rfa, 0.15 to 0.4 Hz, represents parasympathetic function), and LF (Lfa, 0.04 to 0.15 Hz, represents sympathetic function). The HRV and BP are recorded with the patient in sitting position during resting, deep breathing, Valsalva manoeuvre and standing position.

The electrocardiogram (ECG) recordings were initially longer in duration, usually over a period of 24 h but recent data has demonstrated that recording of shorter duration can provide equally reliable information[16,158,161]. Time domain analysis is a useful tool in the assessment of parasympathetic activity by measuring the normal R-R intervals, whereas the frequency domain is based on the spectral analysis of R-R interval and other cardiovascular and respiratory signals based on short-term ECG recordings (2-5 min)[69,158].

The key element in the accurate use and interpretation of HRV models is the standardisation of the conditions under which the test is carried, including age, blood pressure, HR, tobacco smoking or caffeine use and, above all, respiration control[69].

Baro-reflex sensitivity

The BRS measures the cardiac vagal and sympathetic baro-reflex function. The idea behind its function is that an increase in the BP normally induces a reflective in-

crease in the vagal cardiac efferents and a reduction to the efferent sympathetic activity, resulting in bradycardia and hypotension, due to the reduction in cardiac output as well as the peripheral vasodilation[158]. A reduction in BP induces opposite responses. Thus, to correctly measure the baro-reflex function, both the vagal efferent activity (evidenced by changes in HR in response to changes in BP), and the sympathetic efferent activity (affecting the arterial vessels) should be taken into account.

In practice, the term "baro-reflex sensitivity" normally applies to the cardiac-vagal arm, and to methods measuring changes in HR in response to changes in (systolic) BP. The test can be performed either with the use of pharmacological methods (intravenous bolus injection of epinephrine)[162] or non-pharmacological techniques (physical manoeuvres such as postural change). Although the former is considered the gold standard to date for evaluating BRS, both of them correlate well clinically with each other[163]. Both techniques require a continuous measure of BP and a continuous and synchronised measure of HR (R-R interval)[158].

BRS can be used for detecting sub-clinical CAN[63], since BRS can be abnormal in diabetes, before the demonstration of any clinical signs of CAN or other conventional autonomic function tests detect any abnormalities[64,65]. Several studies on patients with diabetes have concluded that BRS is a strong independent risk factor for mortality[164], especially in cohorts suffering from heart failure or following a myocardial infarction[162,165].

Scintigraphy

The use of Single-photon emission computed tomography (SPECT) and/or positron emission tomography (PET) and sympathetic neurotransmitter analogues, such as the ^{123}I-metaiodobenylguanide (^{123}I-MIBG) (SPECT), the ^{11}C-metahydroxyephedrine (^{11}C-HED) (PET) and ^{11}C-epinephrine has enabled the quantitative scintigraphic evaluation of cardiac sympathetic innervation[63].

^{123}I-MIBG undergoes rapid uptake in the myocardium but as it is semi-quantitative is not a precise indicator of neuronal uptake[158]. Metabolically stable ^{11}C-HED demonstrates a highly specific uptake by the sympathetic nerves mediated by norepinephrine transporters[166]. It is important, however, to take myocardial perfusion (which affects the delivery of the tracer of interest) into consideration before interpreting the results of these imaging techniques. Retention defects of both ^{123}I-MIBG and ^{11}C-HED have been reported in patients with T1DM and T2DM and have been variably correlated with abnormal but also normal CARTs[60,67,167]. The consistent pattern of sympathetic denervation in patients with T1DM supports the notion that ^{11}C-HED can be used to monitor the population of sympathetic nerves and evaluate the regional autonomic deficits of sympathetic innervations[66,166,167]. In patients with CAN and T1DM, the wash rates of ^{11}C-epinephrine have been shown to correlate well with those of ^{11}C-HED[158]. The development of microvascular complications has been associated with the augmentation in sympathetic tone and adrenergic hyperresponsiveness, by the use of ^{11}C-HED[63]. As CAN reaches an advanced stage, a heterogenous pattern of ^{11}C-HED retention is observed, with a reduced ^{11}C-HED retention in the distal LV and a persistent or increased ^{11}C-HED retention seen proximally, indicating a proximal to distal pattern of sympathetic denervation of the LV[63].

Increases in the sympathetic nervous tone and elevated epinephrine levels can affect the retention of sympathetic neurotransmitter analogues, making the interpretation of the above scintigraphic models rather challenging. Furthermore, the lack of standardisation, the high cost and the demand on highly skilled operators, restricts the role of scintigraphy as a valuable research tool and not a part of daily clinical routine[68].

When it comes to radiation exposure, ^{123}I-MIBG lacks a β-particle emission and has a half-life of 13.2 h, whereas its energy of the primary imaging photon is calculated at 159 keV (kiloelectron volt)[168]. When compared to ^{131}I, ^{123}I-labelled agent is to be considered the radiopharmaceutical of choice as it has a more favourable dosimetry and better radiation profile. Whole-body radiation is markedly lower using ^{11}C-HED PET [effective dose equivalent in adults, 1.2 milliSieverts (mSv)] compared with ^{123}I-MIBG scintigraphy (effective dose equivalent in adults, 6.0 mSv)[169]. The radiation dose to the whole body from 20 milliCuri (mCi) ^{11}C-HED is 0.186 rad, less than that from 0.5 mCi ^{131}I-MIBG IBG (0.45 rad) or 10 mCi ^{123}I-MIBG (0.53 rad)[170].

Muscle nerve sympathetic nerve activity

Muscle sympathetic nerve activity (MSNA) is based on the ability to record efferent sympathetic nerve signals in the skeletal muscles either at rest or in response to physiological perturbations with the use of microelectrodes into a fascicle or a distal sympathetic nerve of the skin or muscle (microneurography)-usually the peroneal nerve[171].

MSNA is the most direct measure of peripheral sympathetic activity and therefore a useful research tool. However, its invasiveness, cost and time-consuming nature is not recommended for routine autonomic assessment[158].

Other tests

Occasionally, various tests have been proposed for the assessment, diagnosis and monitoring of CAN. A recent study on 167 patients with type I diabetes conducted by the University of Liege, found the use of pulsatile stress, which measures the arterial stiffness, correlates well with baro-reflex sensitivity, suggesting therefore that arterial stiffness can be used as a marker of CAN[172]. The association between arterial stiffness (expressed as carotid-femoral wave velocity (PWV)) had already been explored by another study. After multivariable linear regression, the association between CAN (E/I index in particular) and PWV not only remained significant but E/I index was the strongest predictor of PWV in the model (β coefficient: -0.326, 95%CI: (-3.110)-(-0.750), P = 0.002)[173]. Catecholamine kinetics, most specifically epinephrine and norepinephrine plasma clearance have been labelled as

Significance of CAN

According to the Toronto Consensus Panel on Diabetic Neuropathy statement, *screening for CAN* in the patients with DM should be considered good clinical practice, due to the following:

(1) It enables the accurate and clinical relevant diagnosis of various CAN forms

(2) It assists in the appropriate detection and subsequently the tailored treatment of CAN multiple clinical manifestations as described in the previous section

(3) It provides a clinical tool for the risk stratification for diabetic complications as well as the cardiovascular morbidity and mortality

(4) It can be used for the modulation of targets of diabetes treatment

Figure 3 Current recommendations on screening for cardiac autonomic neuropathy. CAN: Cardiac autonomic neuropathy; DM: Diabetes mellitus.

the biochemical equivalent of MSNA but they have failed to date to produce reliable diagnostic data[158].

Another aspect of autonomic function is the assessment of cutaneous MBF. The skin offer an accessible organ to asses MBF and endothelial function, which is often involved in the development of micro and macrovascular diabetes, correlates with systematic endothelial function measures and myocardial microcirculation[174]. Several methods are available to assess skin MBF[175]. Laser Doppler (LD) allows the determination of blood flow under basal conditions or following physical (*e.g.,* heating) or pharmacological (*e.g.,* acetylcholine and/or sodium nitroprusside) stimulation; allowing the differentiation between endothelial-dependant and independent responses[174]. Furthermore, LD allows the measurement of nerve axon reflex-related vasodilation following acetylcholine iontophoresis which is the result of C-fibre stimulation[176]. LD techniques include LD flowmetry, LD perfusion imaging and laser speckle contrast imaging[158,174].

Another assessment of the peripheral autonomic system is intra epidermal nerve fibre density (IENFD) using immuno staining[177]. IENFD is highly sensitive and specific to diagnose small fibre neuropathy (88%-98% and 88.8%-95% respectively)[178]. IENFD correlates also inversely with thermal thresholds[178]. In addition, IENFD innervates the sweat glands. Reduction in sweat production in the feet contributes to the development of dry skin/callus and hence predispose to the development of foot ulceration. This function can be assessed by several methods such as Neuropad[147] and Sudoscan[179].

CRITERIA FOR DIAGNOSIS AND STAGING

HR responses to deep breathing, standing and Valsalva manoeuvre, as well as blood pressure response to standing (CART) are considered as the gold standard in clinical testing for autonomic neuropathy[10]. Their applicability in bedside clinical practice is based on their sensitivity, specificity, reproducibility, ease and safety of use and standardisation.

According to the CAN Subcommittee of the Toronto Consensus Panel statement following the 8th international symposium on diabetic neuropathy in 2010[10], the criteria for diagnosis and staging of CAN are as follows: (1) A single abnormal CART result suffices for the diagnosis of possible or early CAN; (2) The presence of two or three abnormal test among the seven autonomic cardiovascular indices (5 CARTS, time-domain and frequency-domain HRV tests) are required for the diagnosis of definite or confirmed CAN; and (3) The presence of orthostatic hypotension in addition to the above criteria signifies the presence of severe of advanced CAN.

SCREENING FOR CAN

The majority of diabetes patients with CAN have subclinical or asymptomatic disease, rendering the diagnosis and appreciation of CAN in clinical practice rather difficult[63]. Once CAN reaches the stage that becomes clinically evident, the disease might have reached an advanced level and management becomes more difficult. Screening for early CAN is therefore considered good clinical practice several reasons as summarised in Figure 3[10].

The Toronto Diabetic Neuropathy Expert Group in a recent statement have recommended that screening should be considered for patients at time of diagnosis of T2DM and within 5 years of diagnosis of T1DM, particularly in patients with other macro- and/or microvascular complications[180]. Patients with a history of poor glycaemic control are especially at risk for developing CAN, as demonstrated in several studies, suggesting that this clinical group may benefit from screening[17]. Due to its impact on exercise tolerance, testing for CAN should be a part of the screening in patients that are about to begin a new exercise programme that involves more intense physical activity than brisk walking[69,181]. Evidence also suggests that screening for CAN could be incorporated into the perioperative assessment of patients with poor glycaemic control and coronary artery disease, due to the association between CAN and haemodynamic instability peri- and intra-operatively[182]. Finally, testing for CAN could potentially be of benefit in patients with DM that have suffered MI, as this would serve in the risk stratification of this subgroup and assist into adapting a more aggressive therapeutic approach for those at risk of sudden cardiac death or life threatening arrhythmias.

THERAPEUTIC APPROACHES FOR CAN

CAN treatment can either be symptomatic or aimed at

slowing or reversing CAN progression. However, effective therapies to slow or reverse CAN progression are rather limited as the complete underlying pathogenesis remains unclear. However, based on our current understanding of CAN pathogenesis and risk factors, several potential treatments have been examined.

Lifestyle modification

Lifestyle changes have been shown to have a beneficial impact on the prevention of CAN progression in the Steno-2 trial[5] and the Diabetes Prevention Program (DPP)[183]. In the Steno-2 study, patients with T2DM and microalbuminuria were randomised to a multi-factorial cardiovascular risk factor intervention that included behavioural therapy (diet, physical exercise and smoking cessation) and pharmacological intervention (to control BP, lipids and hyperglycaemia) or conventional treatment in accordance to the national guidelines. After an average of 7.8 years of follow-up, the risk for developing CAN was significantly lower on the intervention arm (49% in the intensive group *vs* 65% in the conventional group, HR = 0.37, 95%CI: 0.18-0.79, $P = 0.002$). In the DPP, lifestyle modification demonstrated superior results in the improvement of autonomic dysfunction (assessed with HRV and QT indexes) as compared to the use of metformin or placebo.

Weight loss and dietary intervention accompanied[69] or not[184] by supervised training was associated with improvement on CAN indices. Aerobic training has also been shown to improve CAN, with some indication that mild physical exercise is recommended in less severe CAN cases. A recent review summarising the evidence for the impact of life style interventions on CAN has concluded that moderate endurance and aerobic exercise in both T1DM and T2DM, improve HRV and cardiac autonomic function significantly, in favour of parasympathetic dominance, independent of BMI, glycaemic or BP control and duration of diabetes[185].

Intensive glycaemic control

Hyperglycaemia is a major risk factor for CAN development and progression. Intensive glycaemic control has been shown to slow the progression and prevent/delay the development of CAN[18,66,186,187]. In the DCCT trial, intensive glycaemic control in a group of patients with T1DM reduced the CAN incidence by 50% over 6.5 years follow-up compared with conventional therapy (7% *vs* 14% respectively)[19]. These beneficial effects persisted 13-14 years after close-out of the trial[18]. Although both former treatment arms exhibited deterioration in CAN during follow-up after the end of the DCCT, the former intensive treatment group continued to demonstrate a statistically significant slower decline in CAN.

PET cardiac imaging with the use of 11C-HED showed similar beneficial effects in a 3-year prospective trial. Good glycaemic control (defined as mean HbA1c < 8%) was associated with reduction of sympathetic denervation as opposed to the group of poor diabetes control (HbA1c ⩾ 8%)[167]. In the SEARCH CVD study, 354 young patients with T1DM were assessed for the presence of sub-clinical autonomic dysfunction, as demonstrated by the use of HRV parameters and the presence parasympathetic loss with sympathetic override. Poor glycaemic control, as defined by HbA1C > 7.5%, was independently associated with the presence of subclinical CAN as compared to a frequency-matched control group without DM[188].

The effects of glycaemic control in T2DM are not similarly encouraging. Data from recent studies have failed to demonstrate differences in the incidence of CAN based on the application of intensive therapy in T2DM patients[189,190]. The sensitivity of tests utilised for the diagnosis of CAN in those trials, however have been questioned, suggesting that more research is needed to investigate the relationship between metabolic control and CAN in patients with T2DM.

Therapies based on CAN pathogenesis

There is limited but increasing data on the use of pharmacotherapy targeting specific pathogenic pathways. The use of the specific antioxidant α-lipoic acid improved CAN in patients with T2DM in a 4-mo controlled randomised trial[191]. In animal models, the pharmacological agents FP15 and FeTMPS, which act by catalysing the decomposition of peroxynitrite, have shown promising results in improving neuronal function[192-194]. The use of glucagon-like peptide 1 analogues or the dipeptidyl peptidase 4 inhibitors have demonstrated cardioprotective[195] and neuroprotective properties[196], raising the possibility of their use for treatment not only for peripheral neuropathy, but autonomic neuropathy as well. In small scale studies, aldose reductase inhibitors have been shown to improve LV function in patients with DAN without any alteration on CAN indices[197]. There is also evidence suggesting the vitamin E and C-peptide can both improve HRV indices[10]. In a randomised controlled trial, vitamin E when compared to placebo managed to increase the R-R interval ($P < 0.05$) and the HF component of HRV (HF; $P < 0.05$) in 50 patients with T2DM over a period of 4 mo[198]. Small RCTs have shown beneficial effect of C-peptide treatment on CAN parameters[53]. In a recent randomised placebo-controlled trial of 44 patients with T1DM, treatment with a triple antioxidant regime (allopurinol, α-lipoic acid and nicotinamide) over the course of 2 years failed to prevent progression of CAN and had no benefit on myocardial perfusion as demonstrated with scintigraphic imaging modalities[199]. Further research is required to confirm these findings and explore other potential pathogenetic therapies.

The renin-angiotensin-aldosterone axis

There is substantial data to support the use of certain pharmacological agents in the improvement of the left ventricular dysfunction associated with autonomic neuropathy in diabetes. In patients with heart failure, the use of bisoprolol[200] or the addition of spironolactone to enalapril, furosemide and digoxin[201], demonstrated a beneficial effect on autonomic function, as shown by

HRV testing and sympatho-vagal balance respectively. The use of angiotensin-converting enzyme (ACE) inhibitors could potentially improve the parasympathetic/sympathetic balance[202] and improve prognosis in cardiac failure[203]. The addition of angiotensin receptor blockers to ACE inhibitors may be superior to monotherapy[204-206], due to the enhanced blockade on the renin-angiotensin-aldosterone axis[207]. In a small study by Didangelos *et al*[208], including 62 patients with type I and type II DM, the use of ACE inhibitors or ARBs, as well their combination, managed to improve both diabetic autonomic neuropathy and LV diastolic dysfunction.

Symptomatic treatment of orthostatic hypotension

Treatment of orthostatic hypotension is required in symptomatic patients with autonomic neuropathy. There are several strategies available, including lifestyle and behavioural measures as well as pharmacological options. The former include advice provided to the patients to avoid sudden changes in body posture, eat smaller and more frequent meals, avoid drugs-precipitants of postural hypotension (diuretics, tricyclic antidepressants, α-adrenoreceptor antagonists), perform physical countermanoeuvres (leg crossing, stooping and squatting), increase fluid and salt intake, avoid physical activity that leads to straining and finally use garments over legs and abdomen[69,209].

If the above measures fail to improve symptoms, pharmacological intervention may be considered. A risk-benefit consideration should take place for each individual before starting a medication, especially weighing up the risk of developing marked supine hypertension against the benefit of preserving the erect blood pressure. Should a pharmacological agent be considered appropriate by the clinician, there are several options available[210-212].

Midodrine, a peripheral selective α_1-adrenergic agonist, is considered a first line agent that acts through peripheral vasoconstriction of arterioles and veins. It remains to date the only drug approved by the food and drug administration (FDA) for the treatment of orthostatic hypotension[213,214]. However, post-market trials to prove drug's efficacy are still ongoing and the final results on midodrine's benefits are scheduled to be published in 2014, 18 years after the drug was given FDA approval[215].

9-α-fluorohydrocortisone, a synthetic mineralocorticoid, is another first line option that acts through sodium retention and plasma expansion[216]. In a double-blinded crossover study, 9-α-fluorohydrocortisone treated successfully the orthostatic hypotension of patients with diabetes and autonomic neuropathy[216]. 9-α-fluorohydrocortisone doses between 100 and 400 micrograms decreased significantly the orthostatic hypotension in 14 symptomatic patients with DM over a mean period of 12 mo ($P < 0.001$)[217]. Extra care should be taken when prescribed in patients with cardiac failure, as it can lead to fluid overload. There is usually a period of 10-14 d before its effects can become clinically evident[212].

Somatostatin and somatostatin analogues (octreotide) inhibit the release of vasoactive peptides from the GI tract and thus increase splanchnic vasoconstriction, leading to increase in mean blood pressure[218]. The use of long acting octreotide in patients with autonomic neuropathy increased the mean systolic BP from 83.8 ± 7.1 mmHg to 104.1 ± 3.1 mmHg ($P < 0.025$) within eight weeks, improving orthostatic dizziness and fatigue[219]. In a study of 18 patients with idiopathic orthostatic hypotension, octreotide reduced postural, postprandial and exertion-induced hypotension, as demonstrated by 24-h ambulatory blood pressure profiles and cusum analyses[220].

Other available pharmacological strategies include the use of erythropoietin which can increase the erect BP through the increase of red cell mass and circulating volume, the improvement of anaemia and its regulatory effect on vascular tone[221] and desmopressin acetate whose efficacy is mainly observed in morning time hypotension[212]. Finally, caffeine and acarbose can potentially be used in the management of post-prandial hypotension[212]. In a case report of 58 years old patient with DM and severe postprandial hypotension refractory to the use of midodrine and octreotide, acarbose (an alpha-glucosidase inhibitor) reduced the postural drop from 50 mmHg to 18 mmHg, improving the patients symptoms dramatically[222].

Unfortunately, despite the different options available, postural hypotension remains a difficult condition to treat and many patients require multiple therapies and develop severe intractable disabling symptoms. Beta blockers might help controlling the tachycardia in some patients[69].

CONCLUSION -SYNOPSIS AND FUTURE CONSIDERATIONS

CAN is very common and is an underdiagnosed complication of DM. CAN is associated with significant increase in morbidity and mortality and plays an important role in the development of diabetic cardiomyopathy and silent ischaemia. Clinicians interpreting exercise tolerance testing should be aware of the reduced accuracy of this test in patients with CAN. In addition, CAN might play a role in the pathogenesis of diabetes-related microvascular complications and the development of lower limb complications. However, before CAN is symptomatic and evident clinically, patients might have sub-clinical CAN for several years. The time scale for the progression from sub-clinical to clinically evident CAN is unknown. In addition, the time scale for the progression from early abnormalities (such as increased LV torsion) to clinically detectable cardiac disease is also unknown. Recent guidelines have recommended screening for CAN in patients with diabetes and issued guidance regarding the criteria used to diagnose CAN. CAN is assessed using several methods including CARTs, HRV, and imaging amongst others. The use of HRV and spectral analysis has simplified CAN testing which nonetheless remains time consuming. Despite our improved understanding of

the pathogenesis of CAN, disease modifying treatment is lacking. Improving glycaemic control, life style changes and CVD risk factors management are the mainstay of treatment, which generally slow the progression of CAN rather than reversing it.

Further research exploring the natural history of CAN and the natural history of the impact of CAN on CVD is needed. Better understanding of CAN pathogenesis is also required in order to develop disease modifying treatments. OSA is increasingly recognised as an important contributor to the development of microvascular complications in DM, hence it is important to clarify the relationship between CAN and OSA as this might identify new treatment targets.

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P- Reviewers: Mitra A, Padwal R
S- Editor: Cui XM **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Platelet thromboxane (11-dehydro-Thromboxane B2) and aspirin response in patients with diabetes and coronary artery disease

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Supported by In part by the Senit Foundation, Scotland (United Kingdom); and grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (Japan)
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Received: December 12, 2013 Revised: February 25, 2014
Accepted: March 11, 2014
Published online: April 15, 2014

Abstract

Aspirin (ASA) irreversibly inhibits platelet cyclooxygenase-1 (COX-1) leading to decreased thromboxane-mediated platelet activation. The effect of ASA ingestion on thromboxane generation was evaluated in patients with diabetes (DM) and cardiovascular disease. Thromboxane inhibition was assessed by measuring the urinary excretion of 11-dehydro-thromboxane B_2 (11dhTxB_2), a stable metabolite of thromboxane A_2. The mean baseline urinary 11dhTxB_2 of DM was 69.6% higher than healthy controls (P = 0.024): female subjects (DM and controls) had 50.9% higher baseline 11dhTxB_2 than males (P = 0.0004), while age or disease duration had no influence. Daily ASA ingestion inhibited urinary 11dhTxB_2 in both DM (71.7%) and controls (75.1%, P < 0.0001). Using a pre-established cut-off of 1500 pg/mg of urinary 11dhTxB_2, there were twice as many ASA poor responders (ASA "resistant") in DM than in controls (14.8% and 8.4%, respectively). The rate of ASA poor responders in two populations of acute coronary syndrome (ACS) patients was 28.6 and 28.7%, in spite of a significant (81.6%) inhibition of urinary 11dhTxB_2 (P < 0.0001). Both baseline 11dhTxB_2 levels and rate of poor ASA responders were significantly higher in DM and ACS compared to controls. Underlying systemic oxidative inflammation may maintain platelet function in atherosclerotic cardiovascular disease irrespective of COX-1 pathway inhibition and/or increase systemic generation of thromboxane from non-platelet sources.

Key words: Diabetes; Cardiovascular disease; Platelets; Thromboxane; Aspirin

Core tip: The effect of aspirin (ASA) on platelet thromboxane (11dhTxB_2) generation in diabetes (DM) and symptomatic cardiovascular disease (CVD) was reviewed. Consistent with a heightened platelet hyperactive background, baseline 11dhTxB_2 was significantly higher in DM and acute coronary syndrome (ACS) than healthy individuals. ASA ingestion inhibited 11dhTxB_2 in all subjects, but there were more ASA poor-responders (ASA "resistant") in DM (14.8%) and ACS (28.7%) than controls (8.4%). Only post-ASA 11dhTxB_2 levels predicted adverse cardiovascular outcomes. ASA poor-

responders had higher isoprostane (8-isoPGF$_{2\alpha}$) levels suggesting an underlying systemic oxidative inflammatory process not affected by ASA that may maintain platelet hyperactivity in DM and atherothrombotic CVD.

Original sources: Lopez LR, Guyer KE, Garcia De La Torre I, Pitts KR, Matsuura E, Ames PRJ. Platelet thromboxane (11-dehydro-Thromboxane B$_2$) and aspirin response in patients with diabetes and coronary artery disease. *World J Diabetes* 2014; 5(2): 115-127 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/115.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.115

INTRODUCTION

Thromboxane A2 (TxA$_2$) is a clinically important prostaglandin metabolite derived from arachidonic acid through the cyclo-oxygenase (COX) pathway with roles in hemostasis and cardiovascular disease (CVD)[1,2]. Platelet enzyme COX-1 converts arachidonic acid into prostaglandin G$_2$ (PGG$_2$), followed by the action of peroxidases into PGH$_2$ and into the biologically active TxA$_2$ by thromboxane synthases[3]. Mainly produced by stimulated platelets, TxA$_2$ behaves as a vasoactive agent that affects blood flow and pressure[4] as well as a pro-thrombotic agent capable of promoting the activation and subsequent aggregation of nearby platelets. The latter function is accomplished by TxA$_2$ binding to thromboxane platelet receptors (TPR), a typical G protein-coupled receptor system with trans-membrane segments. Once bound to TPR receptors, phospholipase C is activated to stimulate cytoplasmic Ca^{2+}-dependent Rho Kinases that activate phospholipase A$_2$ and the up-regulation and expression of glycoprotein complex GP Ⅱb/Ⅲa on the surface of platelets[5,6].

Because TxA$_2$ is the bioactive and clinically relevant pro-thrombotic thromboxane metabolite, it would be the logical choice for testing in the clinical laboratory. However, its high instability and very short half-life (20-30 s) makes the routine measurement technically difficult and impractical. Indeed TxA$_2$ is quickly hydrolyzed into a biologically inactive but more stable thromboxane B$_2$ (TxB$_2$) metabolite[7]. Serum TxB$_2$ may be measured in the laboratory but its concentration can be overestimated due to *ex vivo* platelet activation during blood collection and processing. Other serum factors may also interfere with TxB$_2$ measurements. TxB$_2$ is further metabolized by the liver primarily into an 11-dehydro-thromboxane B$_2$ (11dhTxB$_2$) form. This and other minor stable metabolites like 11dehydro-2,3-dinorTxB$_2$ and 2,3-dinorTxB$_2$ are excreted in the urine (Figure 1). Urinary 11dhTxB$_2$ directly reflects the platelet production of TxA$_2$[8,9], and represents a good and reliable biomarker for the laboratory assessment of platelet activity.

Aspirin (Acetylsalicylic acid, ASA) irreversibly acetylates platelet COX-1 for the entire life cycle of the platelet. Ingestion of low doses of ASA blocks over 95% of platelet COX-1 activity resulting in the inhibition of TxA$_2$ production. For these reasons, ASA is widely prescribed as an aid in the primary and secondary prevention of CVD. Despite its widespread use, not all individuals respond to ASA in the same way[10,11]. In addition, ASA effectiveness is limited because over 15%-25% of patients with arterial thrombosis may develop recurrent vascular events while on ASA treatment. This incomplete ASA response (or poor-responsiveness) to therapeutic doses has been referred to as "aspirin (ASA) resistance", a phenomenon described in healthy populations as well as in patients with diabetes (DM) and CVD. The exact mechanisms responsible for this clinical unresponsiveness remain unclear[12,13].

Currently, ASA is largely prescribed for the primary prevention of cardiovascular events in DM but the evidence supporting its efficacy is surprisingly scarce and controversial[14-16]. Recent observations demonstrate that healthy subjects and DM patients with poor ASA response not only seem to manifest an incomplete inhibition of COX-1, but also display a pro-inflammatory milieu and enhanced oxidative stress[17-19]. On the other hand, diet-induced weight loss in subjects with central obesity reduced platelet reactivity and restored platelet sensitivity to nitric oxide, prostacyclin, and physiologic anti-aggregating agents. High on-ASA Platelet Reactivity (HAPR) has been proposed as a more appropriate term than "ASA resistance" to describe a high platelet reactivity status despite ASA therapy in an individual patient. Further, HAPR has been associated with atherothrombotic events following major vascular procedures and may identify patients at high risk for re-occlusion following percutaneous intervention (PCI) with stenting[20].

11DHTXB$_2$ DETERMINATION AND ASA RESPONSE

There are two distinct groups of tests commonly used to measure platelet activity and response to ASA. The first group is blood-based and relies on platelet aggregation response to exogenous agonists or inhibitors by various means[21]. Because platelet activation or inhibition can be mediated by different receptors and pathways, it is not surprising to see a lack of correlation between the assays[22-24]. The second group of tests consists of serologic or urine-based immunoassays that measure both platelet (COX-1) and non-platelet (COX-2) production of thromboxanes. This discussion will focus on the measurement of urinary 11dhTxB$_2$ as a direct indicator of TxA$_2$ activity and platelet activation. One advantage of this type of assays is that thromboxane production is the primary target of ASA through an effective and irreversible COX-1 inhibition.

11dhTxB$_2$ is a biologically inactive down-stream metabolite of TxA$_2$ with a long (stable) circulating half-life that is readily excreted in the urine and relatively unaffected by *ex vivo* platelet activation and other pre-analytical variables[25,26], hence 11dhTxB$_2$ usefulness as a reliable biomarker to assess platelet activation. Due to its relative

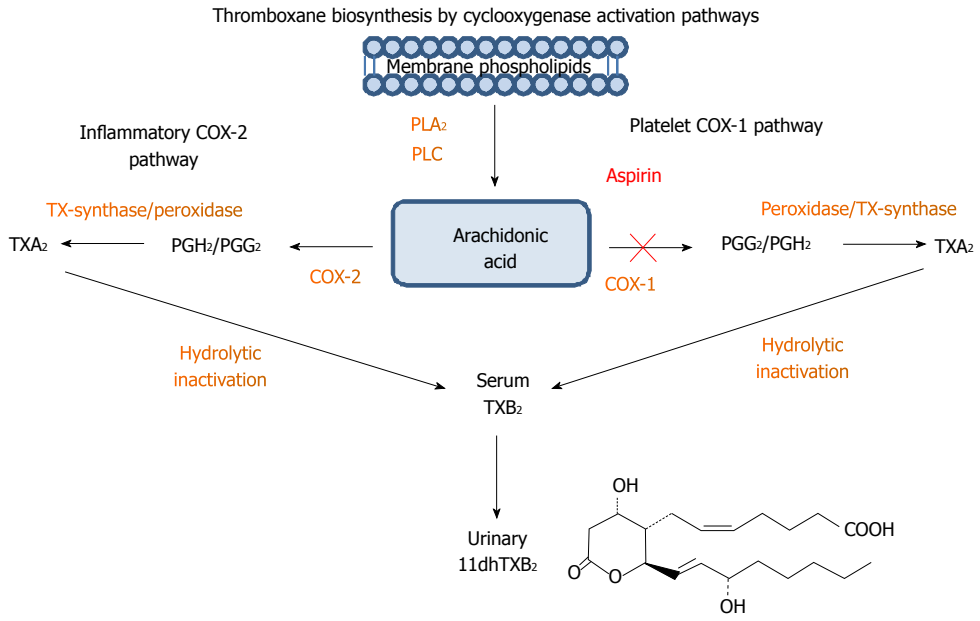


Figure 1 A schematic representation of the arachidonic/thromboxane metabolic pathway: Arachidonic acid generated from membrane phospholipids by phospholipase A2 and phospholipase C undergoes additional enzymatic transformation by cyclooxygenases (COX-1 and COX-2) into prostaglandin and thromboxane metabolites. In platelets, Arachidonic acid (AA) is metabolized by COX-1 into prostaglandins PGG_2, PGH_2 and by thromboxane synthase into the bioactive thromboxane A_2 (TXA_2), which is a potent activator of platelet aggregation with a short half-life. TXA_2 is quickly inactivated into a more stable thromboxane B_2 (TXB_2) and converted in the liver into an 11-dehydro-thromboxane B_2 ($11dhTXB_2$) metabolite excreted in the urine. Aspirin (ASA) irreversibly inhibits platelet COX-1 leading to decreased thromboxane-mediated platelet activation. TXA_2 and $11dhTXB_2$ can be generated by COX-2 present in various inflammatory cells, pathway not affected by ASA.

small size and low concentrations, urinary $11dhTxB_2$ levels are measured by a competitive enzyme-linked immunsorbent assay (ELISA) that uses a spot urine sample without time constraints. Spot urine $11dhTxB_2$ levels are normalized against urine creatinine concentration making the 24 h collection unnecessary. It is important to point out that this ELISA measures the systemic production of thromboxanes (COX-1 and COX-2-derived), and directly reflects COX-1 inhibition by ASA. $11dhTxB_2$ results are first calculated against a reference curve prepared from a reference solution and the final results are reported as pg/mg (pg $11dhTxB_2$ per mg creatinine) to normalize results for urine concentration.

To assess the demographic and clinical variables influencing urinary excretion of $11dhTxB_2$ we first studied apparently healthy adults before and after receiving controlled doses of ASA. Based on the resulting frequency of $11dhTxB_2$ levels, we established a cut-off value to assess an adequate ASA response at 1500 pg/mg of $11dhTxB_2$. This cut-off has been re-confirmed in subsequent studies using both healthy and diseased populations before and after ASA ingestion[27]. Those individuals with urinary $11dhTxB_2$ levels after ASA ingestion below the cut-off of 1500 pg/mg are considered good ASA responders while those with levels above 1500 pg/mg are poor ASA responders ("ASA resistance"). It is important to assess high platelet reactivity in spite of ASA ingestion because a series of actions may be undertaken to manage and reverse the incomplete effect of ASA. The rest of this discussion will focus on a series of clinical studies performed on DM and coronary artery disease (CAD) patients measuring $11dhTxB_2$ and using the quoted 1500 pg/mg cut-off to assess the significance of the ASA response in the development of CVD complications.

ASA poor response or "resistance": definition and clinical implications

ASA "resistance" has been referred to as the lack of a clinical and/or laboratory beneficial effect from ASA ingestion[28-30]. A true or complete ASA "resistance", defined as a lack of response to ASA ingestion due to pharmacologic and/or genetic deficiencies, has not been described to date. The great majority of individuals respond to ASA ingestion as defined by *ex vivo* measurements of platelet aggregation or thromboxane production. However, in most individuals the response seems to be only partial or incomplete. From the clinical point of view, the term ASA "resistance" has neither been fully described nor properly standardized, thus it lacks a nosological clinical definition. Furthermore, consensus guidelines for treatment or management of ASA resistance have not been put forward[31]. Most experts prefer the term ASA "poor or incomplete response" or ASA "insensitivity" to the term ASA "resistance". Throughout this discussion, we will occasionally use the term ASA "resistance" but with the clear understanding that we definitely prefer "poor or incomplete" ASA response as a more appropriate term.

Increased platelet turnover, platelet activation by alternative pathways, alternative/additional sources of TxA_2 production such as macrophage/monocyte COX-2, drug bioavailability, and genetic polymorphisms, have

been implicated in ASA poor responsiveness[13]. Recent reports suggested that CAD patients with high serum concentrations of cholesterol, triglyceride and C-reactive protein had reduced response to ASA measured by platelet aggregation and urinary 11dhTxB$_2$[29]. Compared to asymptomatic patients, those with full blown CAD had significantly higher levels of urinary 11dhTxB$_2$ following ASA ingestion. The HOPE[32] and CHARISMA[33] studies showed that urinary 11dhTxB$_2$ levels in ASA-treated patients predicted the future risk of stroke, myocardial infarction and cardiovascular death. These findings raised the possibility that elevated urinary 11dhTxB$_2$ excretion identifies patients on ASA treatment that are at elevated risk of adverse events and may benefit from additional anti-platelet agents or treatment modification.

Patients who experience a vascular ischemic event while taking ASA have been referred to as having a clinical ASA "resistance". Patients who show a limited inhibition of thromboxane levels, platelet activation, or aggregation after ASA ingestion assessed by biochemical or laboratory tests are referred to as having a laboratory ASA "resistance"[30]. This discussion focuses on the biochemical or laboratory ASA resistance, and more specifically on the clinical impact of the reduced inhibition of COX-1 thromboxane levels. As with any other drug, the dose, drug interference and poor patient compliance should be kept in mind when evaluating ASA responsiveness. The prevalence of laboratory ASA "resistance" ranges from 10% to 25% with occasional peaks up to 60%. However, this wide variability depends on the methods used to measure the ASA response and the patient population under study rather than on the individual response. Nonetheless, other important causes of a poor response to ASA are emerging amongst which is stress-induced inflammation/oxidation[34,35].

An overall poor response to ASA has been associated with up to 13-fold increase risk of atherothrombotic complications in patients with CVD[13,36,37]. A recent meta-analysis of over 20 clinical studies performed on a total of 2930 CVD patients taking ASA (75-325 mg) demonstrated a 4-fold increased risk for any cardiovascular (CV) event including CV death in those patients with poor ASA response[38]. About twenty-eight percent (28%) patients were classified as ASA poor responders ("resistance") suggesting an association with CVD risk. CV-related events were observed in 41%, death in 5.7%, and acute coronary syndrome (ACS) in 39.4% of patients with poor ASA response. It must be pointed out that the clinical studies included in the meta-analysis used different methods to measure platelet ASA inhibition and their own criteria to classify the response to ASA. An interesting observation of the meta-analysis is that ASA poor responders did not benefit from other anti-platelet therapy. The HOPE[32] study screened 5529 patients and measured urinary 11dhTxB$_2$ in 488 ASA-treated CVD patients. Age and sex matched controls also received ASA. CV outcomes including CV death were recorded during a 5-year follow-up. Poor ASA responders with urinary 11dhTxB$_2$ levels in the upper quartile had a 2-fold increasing risk of heart attacks and 3.5-fold risk of CV death. A sub-study of CHARISMA[33] that included 3261 ASA treated CVD patients confirmed the increased CVD risk in patients with 11dhTxB$_2$ in the upper quartile as previously reported by the HOPE study.

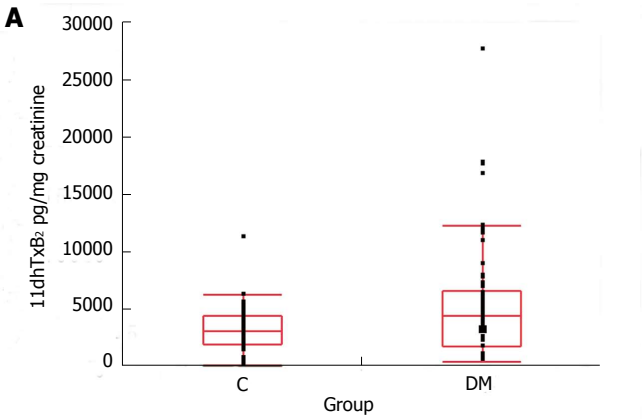
11DHTXB$_2$ AND ASA RESPONSE IN DM

CVD has been long recognized as a leading cause of morbidity and mortality in patients with type 1 and type 2 DM mainly by ischemic heart disease[39,40]. The use of ASA is known to reduce future secondary events in DM[41], however, a meta-analysis of randomized controlled trials failed to demonstrate a clear benefit of aspirin in the primary prevention of major cardiovascular events in patients with DM[42]. To further assess thromboxane levels and aspirin response in DM patients, two clinical studies were conducted on consecutive type 2 DM patients attending the endocrinology and diabetes outpatient clinics in Mexico. The diagnosis of DM was made by the attending physician following internationally accepted diagnostic criteria (World Health Organization DM criteria, 1985) that relied on the presence of abnormal fasting glucose (normal range 70-110 mg/dL), abnormal glucose tolerance test, chronic hyperglycemia and metabolic disturbances of lipid, carbohydrate and protein metabolism due to defects in insulin production or activity. Males and females between 18 and 79 years of age who had not taken ASA or other non-steroidal anti-inflammatory drugs for the previous 2 wk were included. Subjects with liver and kidney disease, symptomatic cardiovascular disease requiring ASA therapy (myocardial infarction, angina, stroke, peripheral artery disease), concomitant acute or chronic inflammatory diseases (bacterial or viral infections), autoimmune disorders, pregnancy, allergy or intolerance to ASA, and bleeding disorders were excluded. The use of ASA in Mexican patients with DM for primary prevention of CVD was significantly less common compared to the US, ensuring a good recruitment of DM patients not taking ASA while avoiding possible unethical discontinuation of the medication.

Baseline 11dhTxB$_2$ levels in DM

Baseline (ASA-free) urinary 11dhTxB$_2$ levels were measured in 100 subjects, 53 with DM and 47 healthy volunteers. None of the patients or controls in this group had received ASA for at least 2 wk prior to testing. The main objective of this study was to establish an average baseline urinary 11dhTxB$_2$ level in DM. The hypothesis was that patients with DM had increased baseline urinary 11dhTxB$_2$ levels hence a higher risk of developing cardiovascular atherothrombotic complication and would receive ASA therapy compared to healthy controls. The mean age of the population studied was 53.9 ± 12.6 years (54 females, 46 males) with mean disease duration of 9.1 ± 7.7 years.

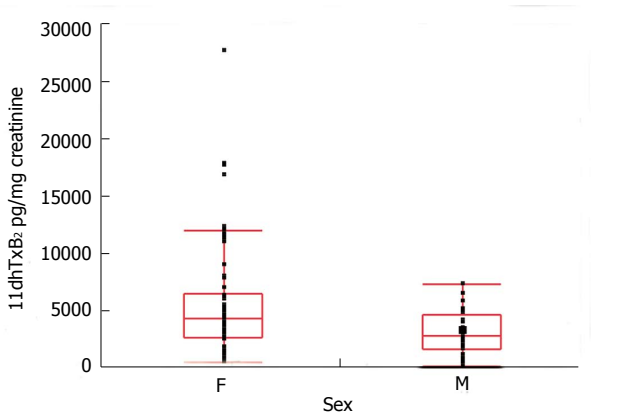
The distribution of baseline (ASA-free) 11dhTxB$_2$ levels of DM patients and healthy volunteers is shown in Figure 2A. DM patients presented with a baseline mean



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars.

Group	11dhTxB2 pg/mg			*P* value[1]
	Mean ± SD	Range	Median	
Diabetes (*n* = 53)	5656 ± 5257	524-27661	4511	0.024
Controls (*n* = 47)	3337 ± 1859	200-11323	3113	

[1]*P* value: Wilcoxon/Kruskal-Wallis test.



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars.

Gender	11dhTxB2 pg/mg			*P* value[1]
	Mean ± SD	Range	Median	
Females (*n* = 54)	5902 ± 5083	524-27661	4364	0.0004
Males (*n* = 46)	2998 ± 1833	200-7333	2891	

[1]*P* value: Wilcoxon/Kruskal-Wallis test.

Figure 3 Distribution of baseline (aspirin-free) urinary 111-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients according to gender. F: Females; M: Males.

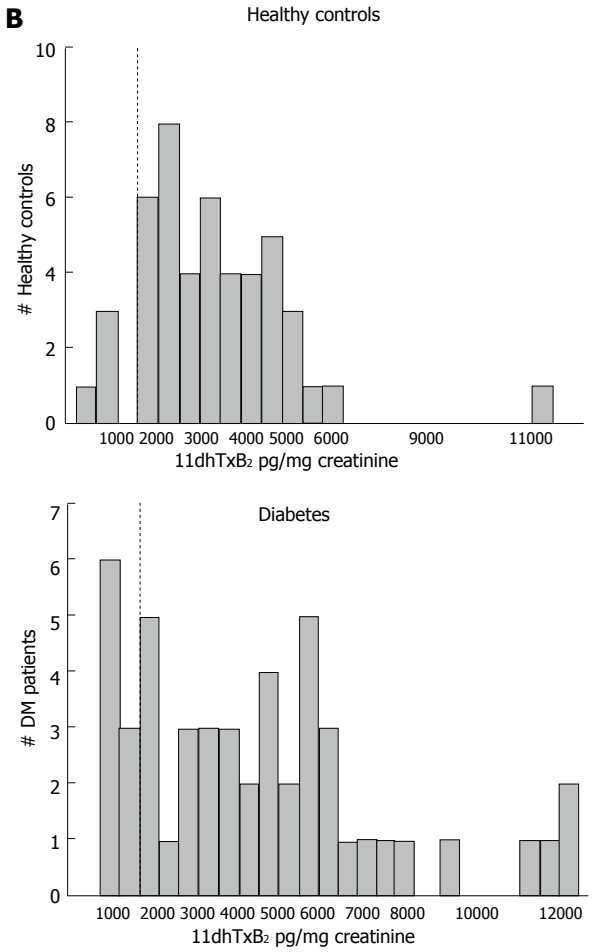


Figure 2 Distribution of baseline (aspirin-free) urinary 11-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients (A, top), and frequency distribution (histogram) of baseline urinary 11-dehydro-thromboxane B2 levels in the two groups studied (B, bottom). A: Comparison of baseline 11-dehydro-thromboxane B2 levels of diabetes and controls; B: Frequency (Histogram) of baseline 11-dehydro-thromboxane B2 levels in diabetes and controls.

urinary 11dhTxB2 excretion of 5656 pg/mg, a value 69.5% higher than the mean baseline 11dhTxB2 excretion of healthy controls at 3337 pg/mg, ($P = 0.024$). The highest 11dhTxB2 value seen in the DM group reached 27661 pg/mg while the highest value in healthy controls was 11323 pg/mg. Figure 2B shows the cumulative baseline frequency of urinary 11dhTxB2 excretion of healthy controls (up) and DM patients (down). The frequency of healthy controls followed a normal (Gaussian-like) distribution while DM patients had a distinctive flat distribution.

Influence of gender, age and disease duration on 11dhTxB2 levels

There were 34 females plus 19 males with DM, and 20 females plus 27 males in the healthy control group. Figure 3 depicts the urinary baseline (ASA-free) 11dhTxB2 levels according to gender. When evaluating all 100 subjects (DM patients and healthy controls), females exhibited a mean baseline urinary 11dhTxB2 excretion 50.9% higher than that of males (5902 *vs* 2998 pg/mg, $P = 0.0004$). When evaluating the influence of gender separately in DM patients and healthy controls, females consistently display significantly higher baseline 11dhTxB2 levels than males (DM $P = 0.01$, controls $P = 0.02$).

The mean age of DM patients was 56 years (range 29-80 years) and that of healthy controls 35 years (range 22-82 years). Figure 4 depicts the association of urinary baseline (ASA-free) 11dhTxB2 levels with the subject's age (in years). In this analysis all 100 subjects (DM patients and healthy controls) were included. The mean disease duration for the DM patients was 9.6 years (range 1-29

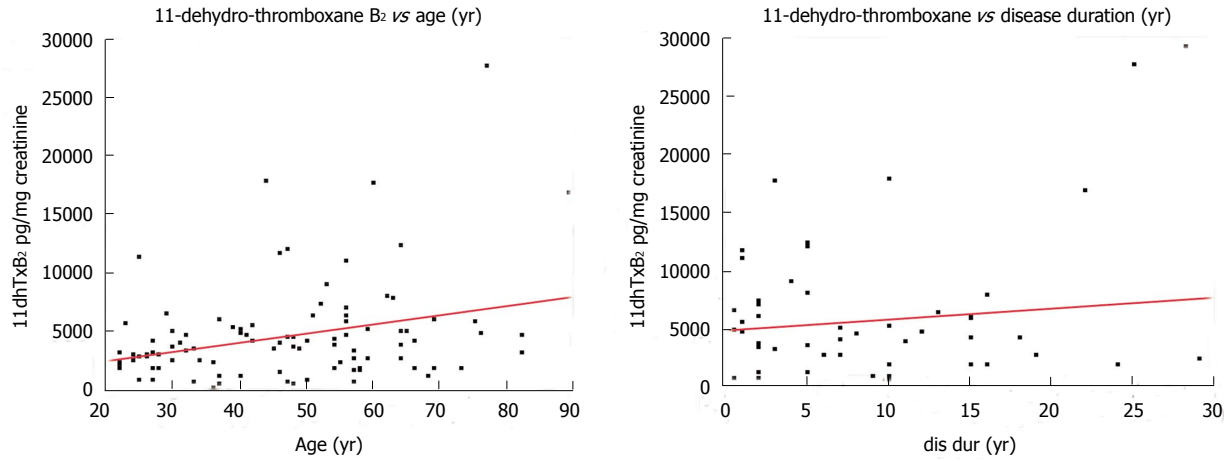


Figure 4 Correlation of baseline (aspirin-free) urinary 11-dehydro-thromboxane B2 levels (pg/mg) measured in healthy individuals and diabetes patients with age (left), and disease duration of diabetes patients (right). Red lines: Linear regression fit.

years). There was weakly positive correlation (r = 0.322, P = 0.001) between age (in years) and baseline urinary 11dhTxB2 levels (left), and a weak (but not statistically significant) positive correlation (r = 0.124, P = 0.3) between disease duration (in years) and baseline urinary 11dhTxB2 levels (right).

These variables were entered into a linear regression model to predict 11dhTxB2 levels (as a dependent variable). Only female gender remained as a significant (P = 0.02) predictor of 11dhTxB2 levels. Sex-related differences in platelet function and aspirin pharmacokinetics in rabbits and man have been previously described[43]. These results support previous findings that ASA reduces the risk of first heart attack in men but not in women suggesting that the ASA effect in women is different[42,44,45]. The results also support the relevance of measuring urinary 11dhTxB2 levels in DM patients to assist health care providers in assessing the risk for CVD and implementing an ASA preventive regimen.

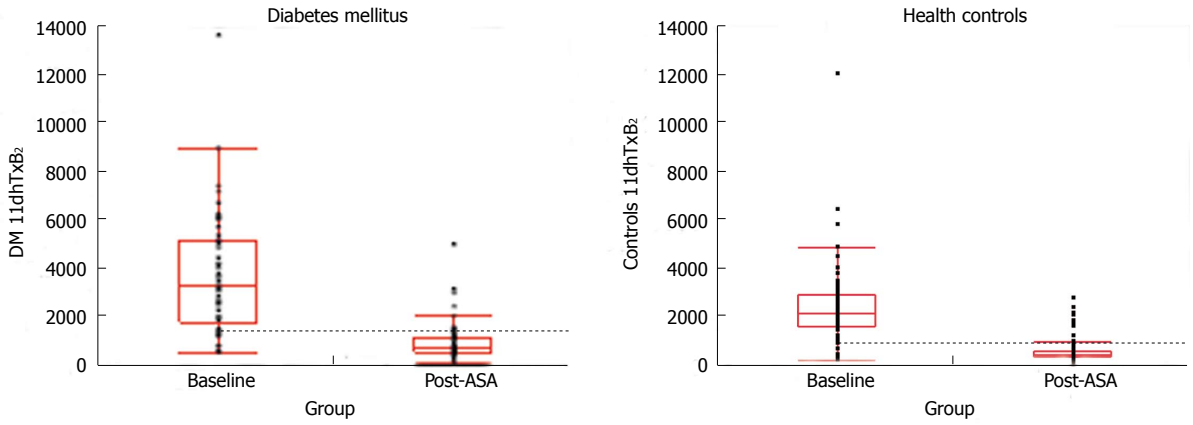
Effect of ASA on 11dhTxB2 levels in DM

The effect of ASA in DM was studied in 137 subjects, 54 patients with DM and 83 healthy volunteers. Each DM patient or control subject contributed two urine samples: one before receiving ASA (baseline) and a second sample after receiving 100 or 325 mg of ASA for 7 d. The main objective of the study was to corroborate that ASA ingestion reduces 11dhTxB2 levels in DM patients. The hypothesis was that ASA would inhibit urinary 11dhTxB2 excretion in DM patients but with more ASA non-responders (11dhTxB2 levels > 1500 pg/mg) compared to healthy controls. The mean age of the final population under study was 54.3 ± 13.1 years with 90 females and 47 males. The mean disease duration was 9.6 ± 7.6 years.

The baseline (ASA-free) and post-ASA ingestion values of DM patients and healthy controls are shown in Figure 5. ASA ingestion suppressed the mean baseline 11dhTxB2 excretion of DM patients by 71.5% (P < 0.0001) as well as the mean baseline of healthy controls (75.1%, P < 0.0001). The baseline 11dhTxB2 excretion of DM patients was greater than that of controls (3664 *vs* 2450 pg/mg, P = 0.001). Similarly, post-ASA 11dhTxB2 excretion of DM patients was greater than that of healthy controls (995 *vs* 624 pg/mg, P < 0.0001). Regarding the effect of the dose of ASA, the mean 11dhTxB2 excretion of subjects taking 100 mg of ASA was 708 pg/mg ± 507, whereas the mean of subjects taking 325 mg was 827 pg/mg ± 811 (P = 0.8). In this study, ASA dose used in DM and healthy controls had no significant influence of post-ASA 11dhTxB2 levels. Furthermore, a regression model to predict 11dhTxB2 levels (as a dependent variable) showed ASA (P = 0.0293) and obesity (P = 0.0467) as statistically significant predictors of 11dhTxB2 levels.

11dhTxB2 excretion shifted below the cut-off (1500 pg/mg) after ASA treatment in the majority of healthy controls, leaving 8.4% (7/83) of subjects classified as non-responders. In DM patients, 11dhTxB2 excretion shifted below the cut-off (1500 pg/mg) after ASA ingestion in the majority of patients, except for 14.8% (8/46) of subjects subsequently classified as ASA non-responders. These results confirm that ASA treatment significantly inhibits baseline urinary 11dhTxB2 levels in both healthy individuals and DM patients, However, there were twice as many ASA poor responders among the DM patients possibly implicating a high platelet reactive phenotype associated with DM [40,46,47].

Having established that DM patients express elevated baseline levels of 11dhTxB2 and twice as many ASA non-responders, we investigated the effect of oxidative stress and anti-oxidant biomarkers on 11dhTxB2 excretion in DM[35]. Urinary 8-iso-prostaglandin-F2α (8-isoPGF2α) and sP-Selectin, nitrite (NO_2^-), nitrate (NO_3^-) and paraoxonase 1 (PON1) activity were measured in baseline (ASA free) and post-ASA samples from these DM patients and controls. Compared to controls, DM expressed increased levels of 8-isoPGF2α (1457 *vs* 1009 pg/mg, P < 0.0001), NO_2^- (11.8 *vs* 4.8 μmol/L, P < 0.0001), NO_3^- (50.4 *vs* 20.9 μmol/L, P < 0.0001) and sP-Selectin (120.8 *vs* 93.0 ng/mL, P = 0.02). ASA demonstrated no effect on 8-isoPGF2α, NO_2^-, NO_3^-, sP-Selectin or PON1 activity in either DM or controls. Again, higher urinary 11dhTxB2 levels in DM suggest a state of heightened platelet acti-



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars. Horizontal broken line represents the 1500 pg/mg cut-off.

	11-dehydro-thromboxane B_2 pg/mg				
Group	Mean ± SD	Range	Median	*P* value[1]	% ASA poor resp
DM baseline (*n* = 54)	3665 ± 2465	508-13578	3255	< 0.0001	
DM post-ASA	996 ± 845	50-5016	693		14.8
Control baseline (*n* = 83)	2450 ± 1572	212-12082	2180	< 0.0001	
Control post-ASA	624 ± 509	37-2834	457		8.4

[1]*P* value: paired *t* test.

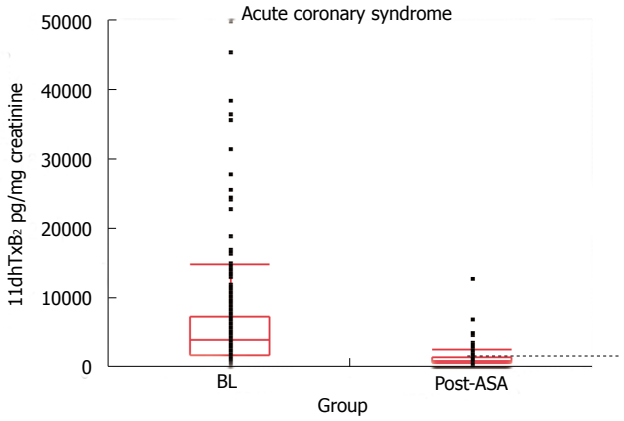
Figure 5 Distribution of baseline (aspirin-free) and post-aspirin urinary 11-dehydro-thromboxane B_2 levels (pg/mg) measured in healthy individuals (right) and diabetes patients (left). 14.8% of diabetes patients were classified as aspirin (ASA) poor responders compared to 8.4% of healthy controls (post-ASA 11-dehydro-thromboxane B_2 over the cutoff 1500 pg/mg).

vation. In addition to platelet hyperactivity, DM patients presented with an inflammatory/oxidative background not affected by ASA. In fact, among the biomarkers measured, only urinary 8-isoPGF$_{2\alpha}$ was significantly higher ($P < 0.009$) in DM patients with poor ASA response. These findings are in agreement with the hypothesis that an oxidative and inflammatory stress may maintain platelet activation irrespective of COX-1 pathway inhibition and/or increase the systemic generation of thromboxane from non-platelet sources *via* COX-2 pathway[34,48-50].

ASA treatment for CVD prevention is a widely accepted practice according to recommended guidelines, but evidence supporting its efficacy is somewhat conflictive and scarce, particularly for patients with DM[51]. The JPAD study (Japanese Primary Prevention of Atherosclerosis with ASA for Diabetes) involved 2539 type 2 DM patients between 40-85 years with no history of atherosclerosis randomized into ASA (81 or 100 mg/d) or non-ASA groups. ASA did not demonstrate a significant reduction in risk for any of the CVD-related endpoints. The POPADAD study (Prevention of Progression of Arterial Disease and Diabetes) included 1276 adults (> 40 years) with type 1 or 2 DM asymptomatic for CVD (ankle-brachial index less than 0.99). ASA (100 mg/d) also failed to demonstrate a significant reduction in risk for any CVD endpoint. Finally, the AAA study (Aspirin for Asymptomatic Atherosclerosis) included 3350 adults (50-75 years) asymptomatic for CVD (ankle-brachial index less than 0.95). ASA (100 mg/d) again did not demonstrate a significant reduction in risk for any endpoint. These studies suggest that DM somehow blunts the beneficial effect of ASA in CVD prevention. Additional mechanisms to explain these clinical findings are forthcoming and likely will help clarify the controversy surrounding the concept of clinical ASA "resistance".

11DHTXB$_2$ AND ASA RESPONSE IN ACS

Two clinical studies of ACS patients will be discussed. One study measured urinary 11dhTxB$_2$ levels after ASA ingestion on 77 consecutive patients attending acute care facilities. ACS patients over 18 years of age undergoing elective PCI at the participating institutions were enrolled. All patients were treated with 325 mg of ASA for at least one week. Each patient provided one urine sample while on ASA. The main objective of the study was to assess urinary 11dhTxB$_2$ excretion in response to 325 mg of ASA in relation to the manufacture's cut-off value of 1500 pg/mg established in apparently healthy individuals. The mean levels of urinary 11dhTxB$_2$ after 325 mg of ASA ingestion was 1550 pg/mg. The majority of ACS patients responded to ASA with 11dhTxB$_2$ levels below the cut-off. However, the percent of ASA non-responders in this ACS population was 28.6%. One common question ponders the dose of daily ASA necessary to inhibit COX-1 and overcome ASA poor response. Urinary 11dhTxB$_2$ levels were measured in 71 consecutive patients with stable CAD and randomized to receive 81 mg, 162 mg and 325 mg per day of ASA for 4 wk. The mean 11dhTxB$_2$ decreased from 931 to 763 pg/mg ($P = 0.046$) with increasing doses of ASA. In this study, the rate of ASA poor responders decreased with increasing ASA dosage. This ASA dose-dependent response is in agreement with previous reports by Gurbel *et al*[22]. Thus,



Box plot: Boxes represent 75/25 percentiles. The horizontal line within the box represents the median for each group. Whisker are 90/10 percentile bars. Horizontal broken line represents the 1500 pg/mg cut-off.

	11-dehydro-thromboxane B_2 pg/mg				
ACS (n = 287)	Mean ± SD	Range	Median	P value[1]	% ASA poor resp
BL (baseline)	7322 ± 13419	86-142691	4242	< 0.0001	
Post-ASA	1349 ± 1110	228-12797	1035		28.7

[1]P value: Paired t test.

Figure 6 Distribution of baseline (aspirin-free) and post-aspirin urinary 11-dehydro-thromboxane B_2 levels (pg/mg) measured in acute coronary syndrome patients. 28.7% of acute coronary syndrome patients were classified as ASA poor responders (post-ASA 11-dehydro-thromboxane B2 over the cutoff 1500 pg/mg). ACS: Acute coronary syndrome; ASA: Aspirin.

ASA dose should be considered when evaluating ASA poor responses.

A second study included 287 consecutive aspirin-free ACS patients admitted to a hospital in Japan for PCI to evaluate a possible association between urinary 11dhTxB2 levels before and after aspirin ingestion with adverse events (AE)[52]. Inclusion criteria included ST elevation myocardial infarction (STEMI), non-STEMI or early onset (within 24 h) invasive revascularization procedure. Upon enrollment and prior to PCI, a baseline (ASA-free) urine sample was obtained, followed by a daily regimen of 100 mg of ASA. Urine samples from ASA-treated patients were collected at hospital discharge (7-14 d) and upon follow up at 6 and 12 mo. Adverse cardiovascular events (AE) were recorded during a 12 mo patient follow-up. Primary end-points included stent thrombosis, Q wave myocardial infarction (QMI), non-QMI, and death (cardiac and non-cardiac). Secondary end-points included stroke, transient ischemic attack (TIA), target lesion revascularization of PCI or CABG, or other vascular event.

The mean age of these ACS patients was 68.9 years. Age did not influence baseline 11dhTxB2 levels (r = 0.060, P = 0.310), but females had significantly higher mean baseline 11dhTxB2 (7675 pg/mg) compared to males (6949 pg/mg, P = 0.0171). The mean baseline ASA-free 11dhTxB2 was 7322 pg/mg for this cohort of ACS patients and was 2-3 times higher than healthy individuals (range 2450-3337 pg/mg). ASA significantly suppressed (81%, P < 0.0001) of baseline 11dhTxB2 levels to 1349 pg/mg at discharge and subsequent time points. The distribution of baseline (before ASA) 11dhTxB2 levels of the ACS patients is shown in Figure 6. In spite of a significant inhibition of 11dhTxB2 by ASA, 28.7% of ACS patients were classified as poor responders by failing to achieve levels below the 1500 pg/mg cut-off. The overall rate of AEs was 17.1%. The rate of AEs according to baseline (ASA-free) 11dhTxB2 levels decreased slightly from 19.4% in quartile 1 to 15.5% in quartile 4. In contrast, the rate of AEs in ASA treatment quartiles increased from 9.1% in quartile 1 to 24.2% in quartile 3 and 20% in quartile 4. The relative risk for AEs of quartile 3 was 2.7 (P = 0.019). When upper quartiles (3 and 4) were compared to lower quartiles (1 and 2), the relative risk was 2.1 (P = 0.011).

High baseline 11dhTxB2 levels were consistent with an underlying platelet hyperactivity that may contribute to the development of atherothrombosis. However, baseline ASA-free 11dhTxB2 levels did not predict 1-year AEs. High levels (> 1500 pg/mg) of 11dhTxB2 after ASA ingestion likely represent extra-platelet (*i.e.*, monocyte/macrophage-derived) COX-2 production of thromboxane. The increased relative risk (2.7) for AEs associated with high post-ASA 11dhTxB2 levels (upper quartiles) suggest that COX-2 production of thromboxane may be a factor associated with a cardiovascular inflammatory process. It is important to point out that ASA insensitive thromboxane generation has been associated with a pro-inflammatory milieu and enhanced oxidative stress in diabetes. Among several biomarkers tested, only baseline urinary 8-isoPGF$_{2\alpha}$ discriminated between normal and poor thromboxane responders, suggesting that oxidative stress may maintain platelet function irrespective of COX-1 inhibition and/or increased systemic generation of thromboxane from non-platelet sources. Thromboxane alone may not be directly implicated in atherothrombosis. Nonetheless, these results confirm previous reports that post-ASA urinary 11dhTxB2 may be useful in predicting adverse outcomes in ACS patients.

Oxidative inflammation (stress) refers to prevailing levels of reactive oxygen species (ROS) in biological systems that overcome their removal by cellular or plasma repair (anti-oxidant) mechanisms[53]. The excess of superoxide anion ($O_2^{\bullet}$) produced by inflammatory cells may exert a free radical attack on cell membranes and/or lipoproteins in a process called lipid peroxidation. While the arachidonic acid metabolism mediated by enzymatic (COX) pathways has received most attention, a non-enzymatic free radical pathway is demonstrating relevance. The free radical oxidation of arachidonic acid generates biologically active F$_2$-isoprostanes reflecting the oxidative status of the organism; is considered a reliable marker of oxidative stress *in vivo*; and has been shown to be an independent risk factor for CAD[54,55]. Some *in vitro* studies have demonstrated that 8-isoPGF$_{2\alpha}$ is capable of stimulating platelet activation while other studies described pro-atherogenic properties through its interaction with the thromboxane platelet receptor (TPR). If 8-isoPGF$_{2\alpha}$ binds to TPR, it may also be capable of competing with TxA$_2$ and activating the Ca^{2+}/Rho kinase

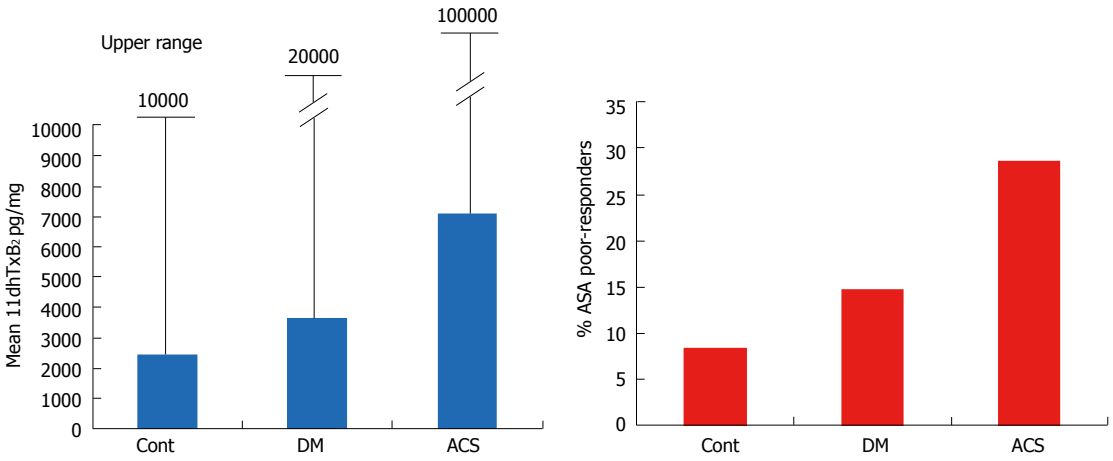


Figure 7 Mean and upper range of baseline (aspirin-free) urinary 11-dehydro-thromboxane B_2 levels (pg/mg) measured in healthy controls, diabetes and acute coronary patients (left), and percent (%) of aspirin poor responders (post-aspirin 11-dehydro-thromboxane B_2 over the cutoff 1500 pg/mg) in the populations studied (right). DM: Diabetes; ACS: Acute coronary syndromes.

pathway[56,57]. This may be particularly important because while TxA_2 enzymatic synthesis is inhibited by ASA, the non-enzymatic 8-isoPGF$_{2\alpha}$ production increases, perhaps as an alternative mechanism to maintain physiologic platelet activity.

Low dose ASA ingestion blocks COX-1 but has no effect on COX-2 or 8-isoPGF$_{2\alpha}$. During an oxidative inflammatory response, increased platelet hyperactivity would come from the combined COX-1, COX 2 and isoprostane (8-isoPGF$_{2\alpha}$) pathways. If ingesting ASA, platelet hyperactivity would be induced by COX-2 and 8-isoPGF$_{2\alpha}$ alone. Limited or no COX-1 TxA_2 production after ASA ingestion would leave unoccupied TPR available to bind 8-isoPGF$_{2\alpha}$ that has a longer half-life (1-10 min *vs* 20-30 s) and higher plasma concentration (351-1831 *vs* 1-66 pg/mL) than TxA_2[6]. Thus, blocking F_2-isoprostane derived from oxidative inflammatory pathways not affected by ASA may be considered in CVD management especially in those individuals with poor ASA response.

CLINICAL SIGNIFICANCE OF 11DHTXB2 MEASUREMENTS

The irreversible inhibition of platelet COX-1 and subsequent reduction of TxA_2 production by ASA has been recognized long ago, making ASA a cost-effective prevention regimen for atherothrombotic CVD. Low doses of ASA have been claimed to prevent over 150000 heart attacks annually. Furthermore, ASA ingestion has accounted for an overall 25% risk reduction of CV events, including a 34% reduction of non-fatal heart attacks, 25% of non-fatal strokes and 18% of all-cause mortality. However, between 25% to 50% of the patients with CAD and ACS did not fully benefit from ASA ingestion[12,13,58]. Thus, TxB_2 measurements to detect those individuals with poor ASA response and higher CVD risk is clinically relevant.

Our studies demonstrated that baseline (ASA-free) urinary 11dhTxB$_2$ excretion showed an upward trend across healthy controls, DM and ACS (Figure 7). The mean 11dhTxB$_2$ of the two control groups studied was 2893.5 pg/mg with an upper range up to 11702 pg/mg. The mean for DM groups was 4660.5 pg/mg with an upper range up to 20619 pg/mg and for ACS patients the mean was 7322 pg/mg with an upper range over 100000 pg/mg. The rate of ASA poor responders had a similar upward trend: controls with 8.4%, DM 14.8% and ACS over 28%.

Baseline 11dhTxB$_2$ levels in both the healthy and diseased populations clearly indicated a wide range of platelet reactivity with a considerable overlap among the groups. This wide range observed likely prevented the establishment of an ASA-free 11dhTxB$_2$ cut-off or even a normal range for clinical use. One relevant observation from the ACS study was that over 40% of ACS patients with high baseline (ASA-free) 11dhTxB$_2$ showed a poor response after ASA ingestion. This observation is in agreement with the concept that higher baseline levels in DM and ACS patients may predict higher rates of ASA poor responders. An explanation for these findings comes from reports that patients with metabolic syndrome (obesity, dyslipidemia, insulin resistance) have increased oxidative stress (oxLDL), higher CVD risk[59], platelet hyperactivity[60] and suboptimal inhibition of platelet COX-1 by aspirin[61], suggesting that higher TxB_2 places these patients at higher risk for thromboembolic events.

Russo *et al*[18] described that diet-induced weight loss in subject with central obesity reduces platelet activation restoring the sensitivity to anti-platelets agents. The Health Aging and Body Composition Study reported that the inflammatory marker interleukin-6 was a robust predictor for new negative health-related events and high urinary 8-isoPGF$_{2\alpha}$ and 11dhTxB$_2$ were associated with higher mortality risk[62]. More recently, Santilli *et al*[63] reported that high intensity physical exercise has broad beneficial effect on platelet activation biomarkers; urinary 11dhTxB$_2$ and 8-isoPGF$_{2\alpha}$ decreased 26% and 21% re-

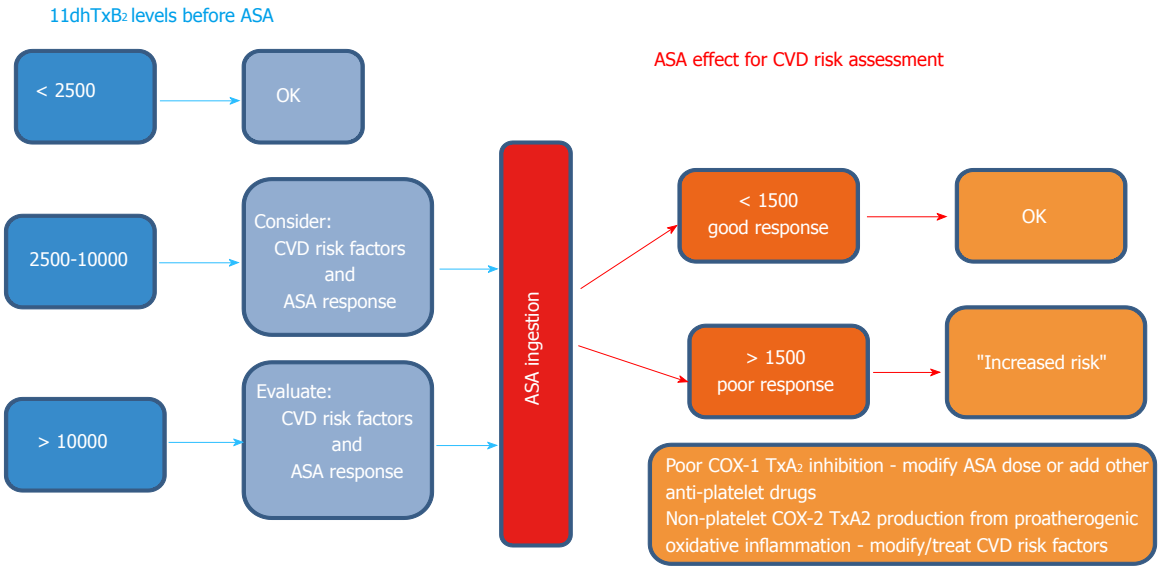


Figure 8 Proposed schematic representation (algorithm) to guide the clinical interpretation and decision making process for assessing CVD risk using baseline (aspirin-free) urinary 11-dehydro-thromboxane B_2 (left), and post-aspirin 11-dehydro-thromboxane B_2 levels (right). Baseline 11-dehydro-thromboxane B2 levels suggested were taken from the mean and upper range of healthy controls. The cut-off of 1500 pg/mg applies only for subjects on aspirin (ASA) therapy to be classified as good or poor responders.

spectively and esRAGE increased 61% compared to the sedentary control group and multiple regression analysis demonstrated that 8-isoPGF$_{2\alpha}$ and esRAGE were the only significant predictors of 11dhTxB$_2$ levels.

The suggestive algorithm discussed below (Figure 8) was developed taking into account the clinical studies discussed above and is proposed to interpret urinary 11dhTxB$_2$ results for CVD risk management.

If the subject is not taking aspirin and the 11dhTxB$_2$ level is

Below 2500 pg/mg: no action is necessary; Between 2500 and 10000 pg/mg: consider giving ASA to assess ASA response and/or consider other underlying CVD risk factors; Over 10000 pg/mg: give ASA to assess ASA response and/or look for other CVD risk factors.

If the subject is taking ASPIRIN and the 11dhTxB$_2$ level is

Below 1500 pg/mg: no action is necessary (good ASA response), continue monitoring CVD risk; Above 1500 pg/mg: the subject is a poor ASA responder ("resistance"). Consider patient compliance, adjusting ASA dosage, additional anti-platelet therapy, *etc*. And more importantly investigate and modify underlying CVD risk factors such as dyslipidemia and inflammatory/oxidative pro-atherogenic background likely responsible for the incomplete inhibition of thromboxanes.

The major impact of this algorithm is that consistently high baseline 11dhTxB$_2$ levels in subjects not taking ASA may justify further investigations for underlying CVD risks. However, only the presence of post-ASA high 11dhTxB$_2$ levels predicts increased risk of atherothrombotic disease. This highlights the need of a comprehensive (multimodal-approach) management that includes both anti-platelet as well as anti-atherogenic treatments.

CONCLUSION

Poor response to ASA frequently indicates an underlying incomplete COX-1 inhibition and increased CVD risk. Among several assays used to measure ASA effect on platelets, urinary 11dhTxB$_2$ reflects systemic production of thromboxanes and platelet reactivity directly affected by ASA. The incidence of ASA poor responders increases in DM and ACS patients, suggesting an active oxidative/inflammatory background likely responsible for both a continued platelet hyperactivity and a pro-atherogenic phenotype not affected by ASA.

Our studies of urinary 11dhTxB$_2$ levels in response to ASA ingestion in diseased populations indicate the following: (1) patients with DM and CAD have significantly higher mean baseline levels of urinary 11dhTxB$_2$ than healthy controls likely indicating a higher platelet activation and risk for CVD. Female gender seems to have a weak positive influence on 11dhTxB$_2$ and platelet reactivity; (2) ASA ingestion significantly inhibited urinary 11dhTxB$_2$ in DM, ACS and controls. However, the rate of DM ASA poor responders (14.8%) was about 2 times higher than controls (8.4%). This may also be a reflection of an increased platelet activation status in DM patients; (3) the rate of ACS ASA poor responders (28.7%) was about 3 times higher than controls; and (4) The results of the studies provide additional support to the laboratory measurement of urinary 11dhTxB$_2$ levels not only in apparently healthy individuals but also in patients with DM and CAD to assess their response to ASA ingestion.

ACKNOWLEDGMENTS

Authors thank Beth L Hurley and Ivana J Muncy for their technical expertise and assistance in testing the samples and organizing the data.

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P- Reviewers: Espino J, Papazoglou **S- Editor**: Song XX
L- Editor: A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (1): Insulin

Impact of hypoglycemic agents on myocardial ischemic preconditioning

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Received: November 27, 2013 Revised: March 13, 2014
Accepted: March 17, 2014
Published online: June 15, 2014

Abstract

Murry *et al* in 1986 discovered the intrinsic mechanism of profound protection called ischemic preconditioning. The complex cellular signaling cascades underlying this phenomenon remain controversial and are only partially understood. However, evidence suggests that adenosine, released during the initial ischemic insult, activates a variety of G protein-coupled agonists, such as opioids, bradykinin, and catecholamines, resulting in the activation of protein kinases, especially protein kinase C (PKC). This leads to the translocation of PKC from the cytoplasm to the sarcolemma, where it stimulates the opening of the ATP-sensitive K^+ channel, which confers resistance to ischemia. It is known that a range of different hypoglycemic agents that activate the same signaling cascades at various cellular levels can interfere with protection from ischemic preconditioning. This review examines the effects of several hypoglycemic agents on myocardial ischemic preconditioning in animal studies and clinical trials.

Key words: Ischemic preconditioning; Myocardial ischemia; Coronary artery disease; Hypoglycemic agents; Diabetes mellitus

Original sources: Rahmi Garcia RM, Rezende PC, Hueb W. Impact of hypoglycemic agents on myocardial ischemic preconditioning. *World J Diabetes* 2014; 5(3): 258-266 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/258.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.258

INTRODUCTION

In the last 3 decades, the prevalence of diabetes mellitus in adults 18 years and older has increased 2-fold[1]. Approximately 50%-60% of patients with diabetes die from cardiovascular disease (CVD)[2]. Among various CVDs, acute myocardial infarction (AMI) has a high rate of mortality, and infarct size is a primary determinant of prognosis in these patients[3-5]. Furthermore, patients with diabetes are more likely than patients without diabetes to develop heart failure after AMI[6]. Thus, the development of new cardioprotective strategies capable of protecting the myocardium are imperative in order to improve clinical outcomes in diabetic patients with coronary heart disease. Moreover, hyperglycemia is an important risk factor for coronary artery disease and death; however, the use of some medications to achieve glycemic control is controversial, as their use has not consistently been shown to reduce mortality. The University Group Diabetes Program (UGDP) in 1970 showed that the administration of tolbutamide, a first-generation sulfonylurea, may increase the risk of cardiovascular death[7].

As a cardioprotective strategy, ischemic preconditioning (IPC) has received much attention for its powerful infarct size-limiting effect. This intrinsic mechanism of profound protection was suggested by Murry *et al*[8] in 1986 who found in a canine model that 4 consecutive periods of coronary occlusion of 5 min were able to reduce

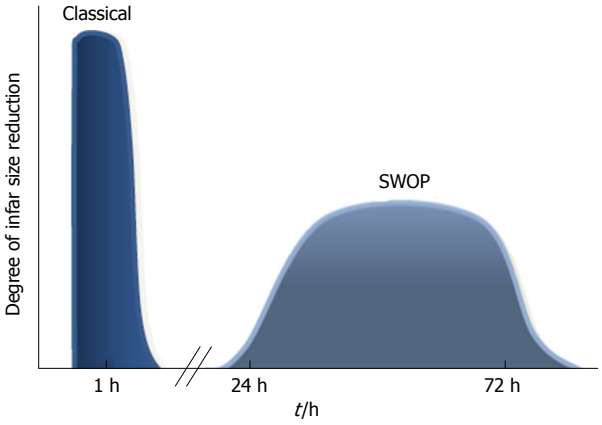

Figure 1 Diagrammatic representation of the temporal nature of the 2 windows of preconditioning (adapted from Baxter *et al*[9]). SWOP: Second window of protection.

the infarct size by as much as 75%, after induction by a subsequent period of occlusion for 40 min. For the first time, it was demonstrated that limitation of infarct size was theoretically possible.

IPC causes 2 phases of protection: "early" or "first window" and "second window of protection" (SWOP). The first window protects the heart for about 2 h and then wanes; the SWOP appears 24 h after the initiation of the IPC protocol and can last for 3 d (Figure 1)[9].

Although IPC was initially referred to as the ability of short periods of ischemia to limit infarct size, some investigators extended this definition to include a beneficial effect on reperfusion-induced arrhythmias[10] and on myocardial stunning[11].

Experimental findings on IPC cannot be directly extrapolated to humans, because of obvious ethical restrictions and because its mechanisms are different from those of other animal species. IPC in human hearts has been demonstrated by results of *in vitro* experiments using human ventricular myocytes[12] and atrial trabeculae[13]. In addition, surrogate clinical endpoints have also been used, including contractile function, electrocardiographic ischemic changes, or biochemical evidence of cell damage.

CELLULAR MECHANISMS OF CLASSICAL PRECONDITIONING

The cellular mechanisms that confer resistance to ischemia have been extensively studied. However, these pathways remain controversial and are only partially understood[14,15]. It has been proposed that endogenous adenosine released during the brief ischemia of the IPC protocol enhances the release of G-protein coupled receptor (GPCR) agonists, such as opioids, adenosine, bradykinin, or catecholamines[16-18]. These GPCR agonists appear to work simultaneously and in parallel to provide redundancy to the preconditioning stimulus. Although these 3 receptors trigger signaling through divergent pathways, this signaling activates prosurvival kinase or reperfusion injury salvage kinase paths, including phosphatidylinositol 3-kinase, protein kinase B, and protein kinase C[14,15]. In turn, it leads to the translocation of protein kinases from the cytoplasm to sarcolemmal receptors[19] and mitochondrial membranes[20], where it phosphorylates a substrate protein, the ATP-sensitive K^+ (KATP) channel[21]. Marinovic *et al*[22] demonstrated in mouse cardiac myocyte cells that the opening of the sarcolemmal KATP channels plays an important role in the prevention of cardiomyocyte apoptosis during metabolic stress, and may interact with mitochondrial channels. Thus, opening of KATP channels are strongly involved in the protection provided by preconditioning[23-26].

Due to the growing knowledge about the cellular pathways of this important protective mechanism, we must consider whether IPC can be applied as a cardioprotective therapy in ischemic heart disease patients.

PHARMACOLOGICAL INTERACTIONS

Pharmacological agents have the capacity to either interfere with signaling or trigger protection. The use of agents capable of mimicking the protective effects of preconditioning, besides brief ischemia, may offer a more benign approach for eliciting cardioprotection. Agents commonly used in coronary disease may interfere with the protection of IPC pathways. Penson *et al*[27] demonstrated in rat-isolated atria and ventricles that activation of beta-adrenoceptors mimics preconditioning. However, β-adrenoceptor blockers impair cardioprotection in animals[28]. Other agents such as Ca^{2+} channel blockers[29] and nonsteroidal anti-inflammatories may interfere with protection by IPC pathways[30,31]. Liu *et al*[16] reported that an adenosine receptor antagonist could block IPC protection and that adenosine or the A1-selective agonist adenosine, instead of brief ischemia, could duplicate IPC protection. Other potential candidates currently in clinical use include nicorandil or diazoxide[32,33]. These drugs have been shown to open KATP channels in ischemic cardiomyocytes, and might act as pharmacological imitators of the preconditioning phenomenon.

HYPOGLYCEMIC DRUGS AND IPC

Hyperglycemia is an important risk factor for coronary artery disease and death. However, the use of some hypoglycemic medications is controversial, because they have not been shown to reduce mortality. Indeed, physicians face challenges regarding the use of new agents in patients with diabetes who are at high cardiovascular risk. Several factors contribute to this concern, and among these is IPC. As described above, the UGDP raised concerns that the administration of tolbutamide may increase the risk of cardiovascular death, but this result remained unexplained until data were reported suggesting deleterious effects of some sulfonylureas (glyburide), specifically in the mechanisms of IPC[23,24].

Insulin secretagogues stimulate insulin secretion by the shutdown of the KATP channel in pancreatic β

cells[34]. KATP channels are composed of 2 types of subunits, inwardly rectifying K^+ channels (Kir6.x) and sulfonylurea receptors (SURx), arranged as tetradimeric complexes (Kir6.x/SURx)[35]. Closure of the KATP channel results in membrane depolarization and influx of calcium (Ca^{2+}) into the β cell. The increase in intracellular Ca^{2+} causes release of insulin from β cell secretory granules. KATP channels are also abundant in both cardiomyocytes[36,37] and arterial smooth muscle cells[38].

The β cell and cardiac muscle KATP channels have been shown to possess a common pore-forming subunit (Kir6.2) but different sulfonylurea receptor subunits (SUR1 and SUR2A, respectively). Although the roles of KATP channel in extrapancreatic tissues are less well characterized, it is likely that they open in response to metabolic stress, such as during cardiac ischemia[39]. Thus, the ideal sulfonylurea for treatment of type 2 diabetes would be one that interacts only with the β cell KATP channel.

EFFECT OF SULFONYLUREAS ON IPC

There is concern about the effect of sulfonylureas on preconditioning protection. Unfortunately, little is known about the ability of the clinically used insulin secretagogues to interfere with IPC. To evaluate studies on the effects of sulfonylureas on IPC, it is important to assess their selectivity for SUR receptor subtypes. These drugs have a range of affinities for KATP channels with different SUR isoform composition, resulting in different abilities to stimulate the KATP channel activity. Tolbutamide has a high affinity for SUR 1 receptors in β cells, but a very low affinity for SUR 2A receptors in the myocardium[40,41]. Glibenclamide (glyburide) inhibits cardiac as well as pancreatic receptors with high affinity[42,43]. Glimepiride has affinity for pancreatic and cardiac SUR comparable to glibenclamide, thereby, does not differentiate between B cells, cardiac muscle, or smooth muscle KATP channels[43,44]. In contrast, preliminary studies reported that glimepiride had less cardiovascular activity than glibenclamide had[45-48]. Several reasons seem to correlate with this finding and, among them, highlight the difference in selectivity for SUR between *in vitro* and *in vivo* studies, and different effects of doses utilized in most studies and in treatment of patients with type 2 diabetes mellitus. In addition, gliclazide, a second generation sulfonylurea, is distinguished by having a higher selectivity for pancreatic SUR receptors[43,49].

Numerous studies using animal models support the hypothesis that IPC is impaired by glibenclamide[23,47,50,51]. Studies using human hearts analyzed IPC in isolated human atrial muscle trabeculae, obtained from type 2 diabetic patients treated with sulfonylureas before coronary artery surgery, and noted that IPC was abolished in patients receiving sulfonylureas[52]. Tomai *et al*[53] evaluated IPC in 20 patients pretreated with either glibenclamide or placebo. They recorded ST-segment changes on ECGs during 2 subsequent episodes of intracoronary balloon inflation. They concluded that human IPC during brief repeated coronary occlusions was completely abolished by pretreatment with glibenclamide. Similar results were shown when the effects of glibenclamide and glimepiride were compared during balloon inflation in percutaneous transluminal coronary angioplasty[45,54].

Tomai *et al*[55] investigated the effects of glibenclamide on the "warm up phenomenon", which is a clinical model of IPC. It refers to an increased tolerance to myocardial ischemia during the second of 2 consecutive exercise tests. In this study, glibenclamide abolished the improvement in ischemic threshold during the second exercise test, compared with placebo[55]. Ovünç[56], in a similar study reported concordant results and suggested that glibenclamide should be used with caution in patients with coronary heart disease and diabetes mellitus, because this agent leads to a decrease in ischemic threshold and exercise capacity. Ferreira *et al*[57], in a study in which IPC was evaluated by 2 consecutive exercise tests, also investigated the effects of chronic treatment with glibenclamide. Forty patients with angina pectoris were allocated into 3 groups: 20 nondiabetic patients, 10 diabetic patients receiving treatment with glibenclamide for at least 6 mo, and 10 diabetic patients receiving other treatments. All patients underwent 2 consecutive exercise tests. The results suggested that IPC protection was blocked in diabetic patients exposed to long-term treatment with glibenclamide. In a recent study, Bilinska *et al*[58] evaluated 64 men, 17 nondiabetic and 47 diabetic, aged 54 ± 5 years. Diabetic patients were allocated into 3 groups: one treated with glibenclamide, one with gliclazide, and the other with diet. All patients performed 2 consecutive exercise tests, with 30 min between them. The authors compared the improvement in ischemic parameters among these groups of patients and concluded that the warm-up effect was preserved in diabetic patients treated with diet, partially preserved in patients treated with gliclazide, and abolished in patients treated with glibenclamide. In contrast, other studies reported no effect of treatment with glibenclamide on the electrocardiographic shifts of the ST-segment during consecutive exercise tests[59,60].

In summary, most studies with glibenclamide (glyburide) reported deleterious effects on IPC, suggesting caution with the use of this agent in patients at high risk for myocardial ischemia.

In animal studies, glimepiride treatment facilitated the cardioprotective effect elicited by IPC[47,48,61-63]. Indeed, data from clinical studies is of great interest. Experimental findings on IPC cannot be directly extrapolated to humans, because in humans its mechanisms are different from those in other animal species. Thus, Klepzig *et al*[45] compared the effects of glibenclamide, glimepiride, and placebo administration on ST-segment shifts during balloon inflation in percutaneous transluminal coronary angioplasty. They concluded that IPC was maintained after glimepiride administration and prevented after glibenclamide. Lee *et al*[46], studied the impact of glibenclamide or glimepiride administration on cardioprotective effects in patients with and without diabetes undergoing coronary angioplasty. The results demonstrated that the

changes in the ST-segment and metabolic parameters were more severe after pretreatment with glibenclamide than with glimepiride, in patients with and without type 2 diabetes.

Only a few studies[45,46] have used IPC protocols in humans to evaluate the effect of glimepiride. To date, these trials have revealed beneficial effects on cardioprotective mechanisms.

In isolated Langendorff perfused rat hearts, the infarct sizes were smaller in the group treated with gliclazide compared with the group treated with glibenclamide. However, the glimepiride group had a smaller infarct size than the gliclazide group[48]. In an *in-vivo* rat study, Maddock *et al*[51] compared the effects of glibenclamide and gliclazide on IPC and nicorandil-induced protection. The IPC protocol consisted of 2 cycles of 5 min of regional ischemia/reperfusion preceding prolonged ischemia. Gliclazide had no adverse effects on IPC or on nicorandil-induced protection. Loubani *et al*[64] assessed the dose-response effect of gliclazide and glibenclamide on IPC. Different doses of glibenclamide and gliclazide were added for 10 min prior to implementation of the IPC protocol. The cardioprotection was abolished by gliclazide only at supratherapeutic concentrations, while glibenclamide prevented IPC at all concentrations.

Bilinska *et al*[58] evaluated the effects of diet, glibenclamide, or gliclazide on the warm-up phenomenon in type 2 diabetic patients with stable angina. They concluded that the warm-up effect was partially preserved in the gliclazide-treated and abolished in the glibenclamide-treated group.

The analysis of the reported data described above suggests that gliclazide does not induce potentially harmful IPC effects.

EFFECT OF GLINIDES ON IPC

The drugs from the glinide class are characterized as insulinotropic agents with a rapid onset and short duration of action. Although glinides do not have a sulfonylurea structure, their role as an insulin secretagogue occurs by binding to the Kir6.2/SUR1 complex, which leads to the closure of KATP channels.

Glinides non-selectively inhibit the pancreatic, myocardial, and non-vascular smooth muscle KATP channels[65]. For these reasons, the selectivity of glinides for the pancreatic compared with the cardiovascular KATP channels has relevance for IPC. Unfortunately, little is known about the ability of the clinically used glinides to interfere with IPC. An original study conducted in our service[66], evaluated the effect of repaglinide on the warm-up phenomenon. Forty-two patients with type 2 diabetes mellitus and coronary artery disease underwent 2 consecutive treadmill exercise tests. After 7 d of receiving repaglinide, 83% of patients no longer had myocardial IPC.

Due to the great difference of *in vitro* selectivity ratios of repaglinide and other drugs in the glinide class (mitiglinide and nateglinide)[43,65], clinical studies assessing the effect of glinides on type 2 diabetic patients with coronary artery disease would be of great interest for both therapeutic and scientific reasons.

EFFECT OF INCRETINS ON IPC

Incretins are gut-derived peptides secreted in response to meals, specifically in the presence and absorption of nutrients in the intestinal lumen. The major incretins are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide. Incretin is mainly represented by GLP-1. The half-life of GLP-1(7-36) in circulation is very brief (1 to 2 min), as it is rapidly degraded by the enzyme dipeptidyl peptidase-Ⅳ (DPP-Ⅳ) to the metabolite GLP-1(9-36), which does not act on the GLP-1 receptor. GLP-1 receptors are expressed in pancreatic islet cells and in the kidney, lung, brain, gastrointestinal tract, and heart[67]. The incretin modulator class includes the GLP-1 analogues or mimetics, which are functional agonists of the GLP-1 receptor. In addition, oral inhibitors of DPP-Ⅳ, in essence, increase the plasma concentrations of the biologically active form of endogenously secreted incretins[68]. Bose *et al*[69] observed in an isolated rat heart model that GLP-1(7-36) is protective against myocardial ischemia-reperfusion injury when given either as a preconditioning mimetic or at reperfusion. Although several investigators have reported the cardioprotective effect of GLP-1, there is a lack of studies about its effects on IPC. Our research group compared the actions of the DPP-Ⅳ inhibitor (vildagliptin) and repaglinide using an IPC protocol. The results showed that vildagliptin preserved IPC in 72% of 54 patients, while repaglinide maintained the cardioprotective response in only 17% of 42 patients[70]. Our group demonstrated 2 effects of hypoglycemic drugs on IPC. These findings support the importance of identifying underlying mechanisms of endogenous myocardial protection to improve the protective effect of pharmacological therapy (Table 1).

EFFECTS OF GLITAZONES ON IPC

The glitazones or thiazolidinediones offer the first therapeutic option specifically directed at reversing the basic problem of type 2 diabetes, which is resistance to insulin. These drugs act on tissues such as liver and skeletal muscle, sensitizing them to insulin action, and thereby increasing glucose uptake and decreasing its hepatic output. The oldest and best-studied glitazone is troglitazone, which was withdrawn from the market by the United States Food and Drug Administration (FDA) because of concerns about its safety. Muriglitazar, which stimulates both PPARγ and alpha receptors, increased adverse cardiovascular events and was also withdrawn by its manufacturer after rejection by the FDA. Roziglitazone and pioglitazone are also drugs in the PPARγ agonist family. Nissen *et al*[71] reported in a meta-analysis a significant increase in the risk of myocardial infarction with rosiglitazone and a trend towards increased risk of death from cardiovascular causes. This information has been includ-

Table 1 Effects of hypoglycemic drugs on ischemic preconditioning

Study	Model	Diabetic drug	Effect
Animal studies			
Gross *et al*[23], 1992	Dogs	Glibenclamide (glyburide)	Abolished
Toombs *et al*[50], 1993	Rabbits	Glibenclamide	Abolished
Mocanu *et al*[47], 2001	Rats	Glimepiride	Preserved
Maddock *et al*[51], 2004	Rats	Glibenclamide	Abolished
		Glimepiride	Preserved
Hausenloy *et al*[61], 2013	Rats	Glimepiride	Preserved
Ye *et al*[62], 2008	Rats	Pioglitazone	Preserved
		Glibenclamide (glyburide)	Abolished
		Glimepiride	Preserved
Horimoto *et al*[63], 2002	Rabbits	Glibenclamide	Abolished
		Glimepiride	Preserved
Bose *et al*[69], 2005	Rats	Native sequenced human GLP-1	Preserved
Zhu *et al*[73], 2011	Rats	Pioglitazone	IPC mimic
Sasaki *et al*[74], 2007	Rats	Pioglitazone	IPC mimic
Ahmed *et al*[75], 2011	Rats	Pioglitazone	IPC mimic
Li *et al*[76], 2008	Rats	Pioglitazone	Preserved
Wynne *et al*[77], 2005	Rats	Pioglitazone	IPC mimic
Sarraf *et al*[78], 2012	Porcine	Pioglitazone	Abolished
		Rosiglitazone	Abolished
Human studies			
Cleveland *et al*[52], 1997	Atrial muscle trabeculae	Glibenclamide (glyburide)	Abolished
Tomai *et al*[53], 1994	Human	Glibenclamide	Abolished
Klepzig *et al*[45], 1999	Human	Glibenclamide	Abolished
		Glimepiride	Preserved
Lee *et al*[54], 2002	Human	Glibenclamide	Abolished
Tomai *et al*[55], 1999	Human	Glibenclamide	Abolished
Övünç[56], 2000	Human	Glibenclamide	Abolished
Ferreira *et al*[57], 2005	Human	Glibenclamide	Abolished
Bilinska *et al*[58], 2007	Human	Glibenclamide	Abolished
		Gliclazide	Partially preserved
Bogaty *et al*[59], 1998	Human	Glibenclamide	Preserved
Correa *et al*[60], 1997	Human	Glibenclamide	Preserved
Loubani *et al*[64], 2005	Right atrial appendages	Glibenclamide	Abolished
		Gliclazide	Preserved (but abolished in supratherapeutic concentrations)
Hueb *et al*[66], 2007	Human	Repaglinide	Abolished
Rahmi *et al*[70], 2013	Human	Repaglinide	Abolished
		Vildagliptin	Preserved

GLP-1: Glucagon-like peptide-1; IPC: Ischemic preconditioning.

ed in the prescribing information for all rosiglitazone-containing products. However, the glitazones have been shown to improve many of the traditional as well as the emerging risk factors associated with CVD[72]. The effect of the glitazones, rosiglitazone, and pioglitazone on IPC is still a matter of debate in the literature, as experimental studies demonstrate contradictory results. Methodological differences are one of the reasons for that. In studies using rat models, pioglitazone was associated with beneficial effects on cardiomyocyte injury, limiting infarct size, and ventricular arrhythmias[73-75]. These beneficial effects may be related to the opening of mitochondrial (ATP)-sensitive potassium channels[76] and by other kinases like phosphatidylinositol 3 kinase and P42/44 MAPK by pioglitazone[77]. On the other hand, in a porcine model, pioglitazone and rosiglitazone had the opposite results[78]. Finally, in the clinical setting, the possible actions of the glitazones on IPC are still uncertain.

EFFECTS OF METFORMIN ON IPC

The cardiovascular benefits observed in diabetic patients with chronic coronary artery disease with the use of metformin[79] have also been observed in experimental studies, which have shown positive results of metformin in the cardiovascular system, and that includes its effect in IPC. It is still not completely understood how metformin protects IPC in the heart, but it is postulated that it activates some kinases involved in IPC, such as (AMP)-activated protein kinase[80], which increases adenosine, activating cardioprotective mechanisms. Recent studies have also demonstrated that metformin increases hexokinase Ⅱ, another important kinase found in mitochondria, which seems to be one of the end-effectors of IPC, and that ultimately protects many cell types, including cardiomyocytes, against apoptosis and ischemic cell death[81]. Ischemia inhibits the loss of hexokinase Ⅱ from mitochondria, consequently preventing the opening of the mitochondrial permeability transition pore. This pore is responsible for the stabilization of the mitochondrial membrane potential, the prevention of cytochrome C release and also the reduction in reactive oxygen species production, which all finally lead to mitochondrial protection against ischemic injury[82,83]. These actions

associated with metabolic alterations, such as the prevention of acidosis through enhanced coupling of glycolysis and glucose oxidation and inhibition of fatty acid oxidation[81], are the responsible pathways by which metformin protects the myocardium from ischemia, in addition to its well-known effects in glucose control.

CLINICAL IMPLICATIONS

Ischemic preconditioning is a complex, dynamic phenomenon that can be the target of drug activities affecting the heart's ability to adapt to ischemic stress. In the clinical setting, however, the literature contains conflicting results regarding whether the use of conventional oral hypoglycemic agents affect cardiovascular mortality[84-90]. The findings from studies about the effects of hypoglycemic drugs on IPC have implications for diabetic patients, especially for those with a high risk of myocardial ischemic events, because the results infer that the myocardium may or may not benefit from a cardioprotective response when under the influence of such drugs. The most important consideration in this matter is that therapeutic options for diabetes treatment go beyond glucose-lowering efficacy in populations with increased risk of coronary ischemic events, and further large clinical trials will be necessary to determine whether the interference with myocardial preconditioning translates into clinical evidence.

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P- Reviewers: Gao GF, Simkhovich BZ **S- Editor**: Wen LL
L- Editor: Cant MR **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diabetes, sleep apnea, obesity and cardiovascular disease: Why not address them together?

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Received: November 26, 2013 Revised: March 1, 2014
Accepted: May 16, 2014
Published online: June 15, 2014

Abstract

Obesity, sleep apnea, diabetes and cardiovascular diseases are some of the most common diseases encountered by the worldwide population, with high social and economic burdens. Significant emphasis has been placed on obtaining blood pressure, body mass index, and placing importance on screening for signs and symptoms pointing towards cardiovascular disease. Symptoms related to sleep, or screening for sleep apnea has been overlooked by cardiac, diabetic, pulmonary and general medicine clinics despite recommendations for screening by several societies. In recent years, there is mounting data where obesity and obstructive sleep apnea sit at the epicenter and its control can lead to improvement and prevention of diabetes and cardiovascular complications. This editorial raises questions as to why obstructive sleep apnea screening should be included as yet another vital sign during patient initial inpatient or outpatient visit.

Key words: Obstructive sleep apnea; Diabetes; Obstructive sleep apnea screening; Obstructive sleep apnea; Cardiovascular complications

Core tip: Obesity, diabetes, cardiovascular disease and obstructive sleep apnea are one of the most common chronic diseases involving population globally. Efforts have been directed towards prevention and public education about the disease process of each of this condition separately. Though these diseases are interlinked, but educational efforts are failing short to address them together.

Original sources: Surani SR. Diabetes, sleep apnea, obesity and cardiovascular disease: Why not address them together? *World J Diabetes* 2014; 5(3): 381-384 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/381.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.381

OBSTRUCTIVE SLEEP APNEA

Should obstructive sleep apnea (OSA) screening be included as yet another vital sign during the patient first visit? Obesity and metabolic syndromes are emerging as major public health issues. One point one billion adults population worldwide are overweight, and approximately 312 million of them are obese[1]. Obesity is highly prevalent in United States but the prevalence is increasing in China, Southeast Asia, Middle East and Pacific Island[2]. The increasing incidence of childhood obesity and its association with the cardiovascular disease is also becoming a major public health concern[3,4]. The number of individuals inflicted with diabetes worldwide is approximately 285 million, but is expected to increase to 439 million by 2030[5]. 17 million deaths out of 57 million total worldwide deaths are attributable to cardiovascular disease[6]. The prevalence of OSA is between 4%-7% and increasing[7].

Obesity and OSA seem to be an epicenter for most of the chronic disease catastrophe. OSA is one of the most common diseases, with a high incidence and preva-

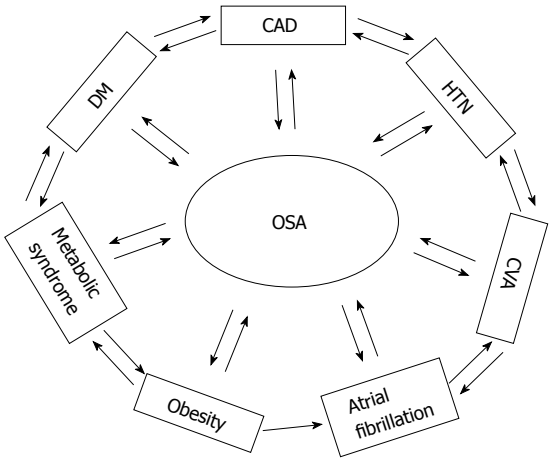

Figure 1 Showing the relationship of obstructive sleep apnea to cardiovascular diseases, diabetes, metabolic syndrome and obesity. CAD: Coronary artery disease; HTN: Hypertension; CVA: Cerebrovascular accident; DM: Diabetes mellitus; OSA: Obstructive sleep apnea.

lence rate that parallels with increasing obesity globally. OSA can be seen in non-obese patients with craniofacial abnormality and children with enlarged tonsils and adenoids too[8-10]. The growing prevalence of obesity and the increasing population body mass index has created major public health challenges[11]. Obstructive sleep apnea has been independently linked with hypertension, atrial fibrillation, cardiac disease, worsening of diabetes, insulin resistance, peri-operative and postoperative complications and coronary artery disease (CAD), to name the few[12-16]. In other words, the data links obstructive sleep apnea to a majority of chronic illnesses. In addition to the illness, untreated OSA increases the health care utilization, impairs work place efficiency, occupational injuries and increase healthcare utilization leading to billions of dollars in economic burden worldwide[17]. OSA if recognized can be adequately treated by an armamentarium of several different treatment modalities. Despite that 85% of the patients with clinically significant and treatable OSA have never been diagnosed, in other word the data has not made to the bedside[18].

OSA involves partial or complete collapse of the upper airway, despite respiratory efforts alternating with normal breathing. It affects 4%-7% of the population[7] and its prevalence in patients with cardiovascular disease is very high. Apnea is defined as a decline in peak signal excursion by $\geqslant$ 90% of their pre-event baseline for $\geqslant$ 10 s. Hypopnea is defined as a drop in the signal excursion by $\geqslant$ 30% of their pre-event baseline for $\geqslant$ 10% and $\geqslant$ 3% arterial oxygen desaturation or accompanied by an arousal[19]. OSA severity is based on Apnea-hypopnea index/h (AHI/h) It can be divided into mild OSA (AHI 5-15/h), Moderate OSA (AHI 15-30/h), and severe OSA (AHI > 30/h). The pathophysiology of obesity and OSA is intimately linked together. Obesity is a major risk factor for OSA. In obese patients there is an enlargement of soft tissue structures in the upper airway, leading to airway obstruction, especially during rapid eye movement sleep when there is atonia. In addition to obesity, there is an increase in fat deposition under the mandible, macroglossia, and palate, which can then lead to narrowing of airway and lead to apnea and hypopnea[20,21]. Obesity has been linked as the central and reversible cardiovascular risk factor that positively influences OSA, diabetes mellitus (DM), metabolic syndrome, hypertension, and lipid metabolism[17]. Children are not immune to the obesity, as the prevalence of obesity among children aged 2-5 is 10% and 6-19 years old is 15%[22].

OSA affects an estimated 15 million adult Americans, especially patients with hypertension, Atrial fibrillation (A-Fib), CAD, and congestive heart failure (CHF) where it is pervasive and levels are very high[23]. Additionally, OSA treatment has also been shown to improve atrial fibrillation incidence, coronary stent reclogging, and improvement of CHF and improvement in blood glucose and insulin resistance[24-29]. Recent evidence directly links OSA and obesity to CAD, heart failure, cardiomyopathy, A-Fib and DM and they are interrelated too as shown in Figure 1. The rise of obesity and DM has been an increased threat to the health of the global population, which has been catalyzed and compounded by the increased occurrence of OSA. In a recent study by Sleep AHEAD Research Group, OSA (AHI $\geqslant$ 5) was found to be in 86% of the population, whereas the pervasiveness of all forms of cardiovascular disease was 14%[30]. On the other hand, individuals who have DM and metabolic syndrome have an increased risk of cardiovascular disease and stroke[31].

The screening for OSA for commercial drivers has been suggested by several societies as American College of Chest Physician, American College of Occupational and Environmental Medicine, and National Sleep Foundation. The International Diabetes federation also recommends screening patients for possible OSA[32]. This screening among the commercial drivers has been successfully implemented, on the other hand, peri-operative screening has been suggested but not implemented in majority of the hospitals despite the availability of simple screening tools as STOP-Bang Questionnaire[33], Berlin Questionnaire[34], neck size, airway, morbidity, Epworth Sleepiness Score, snoring (NAMES) criteria, all with the sensitivity ranging from 80% to 86%[35].

This data has been in literature now for several years, indicating the associations of OSA with almost any disease as glaucoma, end stage renal disease, chronic obstructive pulmonary disease, polycystic ovarian syndrome, metabolic syndrome, cardiovascular disease, stroke, depression, obesity and DM. Moreover, the treatment has led to improvements in the underlying condition[36-38]. The screening test carries high sensitivity, but also has a low specificity. This can result in a plethora of false positive diagnosis and may increase the health care cost. There is high relationship between OSA, hypertension, cerebrovascular disease, CAD and A-Fib. Early diagnosis and treatment of OSA will help in preventing the increase morbidity and mortality associated with those conditions. Studies have shown the improvement in ejection fraction, carotid intimal thickening and benefits in

coronary artery disease, maintenance of sinus rhythm from A-Fib after cardioversion and improvement in insulin resistance. Moreover untreated OSA is also associated with increased risk of death[39-46]. The question arises, if it is the prime time to push for OSA screening for every patient walking in outpatient clinic or hospital? Or do we have to adjust the cutoff of points of our screening test so we can compromise with a decrease in sensitivity to have better specificity to avoid excess healthcare cost as a result of high false positive tests. It is the opinion of the author that Stop-Bang questionnaire, Berlin or NAMES questionnaire can be utilized as the screening tool. In the presence of symptoms, patient should undergo formal sleep study with home sleep study or overnight in lab polysomnography[33-35]. Regardless, one thing is clear: that every physician, nurse and midlevel provider needs to educate patients on risk prevention and education regarding the causes, signs and symptoms of diabetes, sleep apnea, obesity prevention and cardiovascular disease prevention. It is about time that health care providers take the responsibility of preventative education of such diseases as a package rather than fragmentation of education of diabetes in diabetic clinics, sleep apnea in sleep clinics, and cardiovascular disease in heart clinics, as these diseases are interrelated. I will leave the debate open as to if it is about time to push for screening of OSA as one of the vital signs on every patient initial visit.

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P- Reviewers: Cho CE, Nakashima T, Skobel E, Teragawa H, Unal M
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart

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Author contributions: All authors contributed equally to this article.

Supported by Grants from the American Heart Association, No. SDG5310006 (JAS) and No. BGIA4150122 (JAS)

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Received: August 29, 2014 Revised: September 23, 2014
Accepted: October 31, 2014
Published online: December 15, 2014

Abstract

Chronic hyperglycemia is one of the main characteristics of diabetes. Persistent exposure to elevated glucose levels has been recognized as one of the major causal factors of diabetic complications. In pathologies, like type 2 diabetes mellitus (T2DM), mechanical and biochemical stimuli activate profibrotic signaling cascades resulting in myocardial fibrosis and subsequent impaired cardiac performance due to ventricular stiffness. High levels of glucose nonenzymatically react with long-lived proteins, such as collagen, to form advanced glycation end products (AGEs). AGE-modified collagen increase matrix stiffness making it resistant to hydrolytic turnover, resulting in an accumulation of extracellular matrix (ECM) proteins. AGEs account for many of the diabetic cardiovascular complications through their engagement of the receptor for AGE (RAGE). AGE/RAGE activation stimulates the secretion of numerous profibrotic growth factors, promotes increased collagen deposition leading to tissue fibrosis, as well as increased RAGE expression. To date, the AGE/RAGE cascade is not fully understood. In this review, we will discuss one of the major fibrotic signaling pathways, the AGE/RAGE signaling cascade, as well as propose an alternate pathway *via* Rap1a that may offer insight into cardiovascular ECM remodeling in T2DM. In a series of studies, we demonstrate a role for Rap1a in the regulation of fibrosis and myofibroblast differentiation in isolated diabetic and non-diabetic fibroblasts. While these studies are still in a preliminary stage, inhibiting Rap1a protein expression appears to down-regulate the molecular switch used to activate the ζ isotype of protein kinase C thereby promote AGE/RAGE-mediated fibrosis.

Key words: Type 2 diabetes mellitus; Cardiac fibrosis; Fibroblasts; Advanced glycation end product; Rap1a; Extracellular matrix

Core tip: Chronic hyperglycemia is a characteristic of diabetes and one of the major causal factors of diabetic complications. In type 2 diabetes mellitus, mechanical and biochemical stimuli activated profibrotic signaling cascades resulting in myocardial fibrosis, impaired cardiac performance, and ventricular stiffness. Glucose nonenzymatically reacts with extracellular matrix (ECM) proteins forming advanced glycation end products (AGEs). AGE-modified collagen increases matrix accumulation and stiffness by engaging the receptor for AGE (RAGE), the receptor for AGE. To date, our understanding of the AGE/RAGE cascade remains imprecise. This review discusses the AGE/RAGE signaling cascade and proposes an alternate role for Rap1a in diabetic cardiovascular ECM remodeling.

Original sources: Zhao J, Randive R, Stewart JA. Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart. *World J Diabetes* 2014; 5(6): 860-867 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/860.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.860

INTRODUCTION

Chronic hyperglycemia is one of the main characteristics of diabetes mellitus. There are two forms of the disease, which are classified based upon insulin dependence: type 1 diabetes mellitus (T1DM) or T2DM. T1DM is considered a progressive autoimmune disorder of the pancreas causing the destruction of islet β-cells and resulting in diminished insulin production. The subsequent insulin deficiency results in elevated blood glucose levels. T2DM is generally coupled with metabolic syndrome, which includes increased insulin resistance, hyperglycemia, obesity, dyslipidemia and hypertension. Persistent exposure to elevated glucose levels has been recognized as one of the major causal factors of diabetic complications resulting in pathologies, such as atherogenesis, myocardial infraction, stroke and diabetic cardiomyopathy[1]. In this review, we will discuss one of the major fibrotic signaling pathways, the advanced glycation end product (AGE)/the receptor for AGE (RAGE) signaling cascade driven by chronic hyperglycemia in T2DM, as well as propose an alternate pathway that may offer insight into cardiovascular extracellular matrix (ECM) remodeling.

FIBROBLAST MEDIATED ECM REMODELING

In the heart 70%-80% of the cellular mass is composed of myocytes, and the remaining 20%-30% the total cell number includes fibroblasts, vascular smooth muscle cells, and endothelial cells[2,3]. Fibroblasts are the most abundant cardiac cell types of the latter group, and these cells are accountable for homeostatic upkeep and pathological ECM alterations observed in the heart[2,3]. Fibroblasts also function as sensory cells recognizing mechanical and chemical changes within the cell's microenvironment[4]. Fibroblasts communicate with the surrounding ECM to maintain the structural arrangements of the heart as well as sustain vital cellular tasks, such as viability, proliferation, and motility[5].

In pathologies, like T2DM, where biochemical and mechanical stimuli alter the communication between the ECM and fibroblasts, profibrotic signaling cascades are subsequently activated to elevate fibrotic accumulation and subsequently increased heart stiffness[4,6,7]. Increased ECM deposition and accumulation may result from either enhanced matrix protein synthesis and/or decreased structural degradation. With elevated matrix production and accumulation structural ECM rearrangements would cause alterations in fibroblast-matrix interactions. These changes often result in transformations in fibroblast phenotype. Fibroblast isolates from hypertensive animals as well as from infarcted regions of the heart exhibit increased matrix production and accumulation, reduced cell migration, and greater contractility[8-10]. In these instances, changes in fibroblast phenotype correspond to increases in fibroblast to myofibroblast differentiation. Myofibroblasts are defined as a "stressed" fibroblast having increased matrix production as well as enhanced contractile properties[11-13].

This cell type is not commonly found in healthy myocardium, however upon pathological cardiac injury, myofibroblast populations will increase in the myocardium from differentiated interstitial and adventitial fibroblasts[13]. While initially beneficial in pathologies requiring enhanced scar formation to maintain organ integrity (*e.g.,* myocardial infarction), myofibroblasts become detrimental to organ function if an increased population of myofibroblasts persists. Due to the high glucose levels seen in diabetic patients, studies have demonstrated an elevated synthesis and accumulation of the ECM, otherwise known as fibrosis, to increase ventricular stiffness to negatively impact heart function[14,15]. Ultimately, myofibroblasts are detrimental due to their critical role in cardiac pathology and remodeling, and in certain environments, such as diabetes mellitus, improper regulation of myofibroblasts leads to maladaptive tissue remodeling[13,16].

HYPERGLYCEMIA AND AGE

Numerous reports have documented chronic hyperglycemia is the causative agent responsible nonenzymatic formation of AGEs on substrates resistant to turnover, such as collagen[13]. These modifications will not only reinforce the ECM by adding surplus collagen structural crosslinks but also as a RAGE agonist. Chronic hyperglycemia, as observed in T2DM patients, increases the generation of AGEs. High levels of glucose nonenzymatically react with long-lived proteins forming reversible Schiff base intermediates and eventually, Amadori compounds[17]. Amadori products will undergo additional chemical alterations to be converted to nonreversible crosslinked AGES[17]. AGEs are also found to accumulate in normoglycemic patients as a result of longevity. Under high glucose settings observed in diabetics, AGE formation is accelerated, resulting in cardiac dysfunction as well as interstitial fibrosis[17-20]. AGE-modified collagen causes an increase in matrix stiffness causing it be resistance to hydrolytic turnover, resulting in an accumulation of ECM[17,21].

In vivo and *in vitro* studies demonstrate that AGEs account for many of the diabetic cardiovascular complications through their engagement of RAGE[22]. RAGE is capable of binding to multiple ligands. Under normoglycemic conditions the receptor is ordinarily expressed at reduced basal levels, however due to aging and to chronic hyperglycemia, RAGE expression is increased[17,20]. AGE/RAGE cascade activation promotes fibrosis growth factor secretion, increased matrix deposition progressing to multi-organ fibrosis, as well as increased RAGE expression[21,23-25]. Increased AGE crosslinks, AGE/RAGE cascade activation, and increased matrix accumulation have been correlated with the development of cardiovascular complications by increasing diastolic left ventricular stiffness[21,25,26]. AGEs have been demonstrated to increase expression of multiple collagen types, decrease proteo-

glycans synthesis, as well as generate ECM crosslinking. Interestingly, AGEs can be bound to other macromolecules to compound their negative impacts on a number tissues[15,27,28]. Also, they have been shown to perturb cell-matrix interactions, alter cell adhesion, and vascular permeability. Many of the maladaptive ECM alterations have been shown to be relatively corrected by disrupting the AGE/RAGE signaling cascade[29]. Therefore, the AGE/RAGE cascade provides a hypothetical focus for the management of diabetes-mediated ECM related cardiovascular diseases.

AGE/RAGE SIGNALING PATHWAY

Increased AGE/RAGE signaling has been demonstrated to promote key pathways that upregulate ECM protein expression and accumulation. In addition, activation of downstream signaling kinases such as p38, extracellular signal-regulated kinase 1/2 (ERK 1/2), nuclear factor-kappaB (NF-κB), and c-Jun N-terminal kinase (JNK), have been shown to mobilize multiple transcription factors to stimulate expression of growth factors and ECM protein accumulation[30-33]. Numerous studies have suggested that AGE/RAGE signaling pathways are ligand- and cell type dependent. For example, in endothelial progenitor cells, AGE/RAGE cascade activation inhibited migration while promoting apoptosis to further atherosclerosis in diabetic patients[34,35]. Upon treatment with anti-RAGE peptide antibodies, AGE/RAGE signaling pathway was down regulated and diabetic atherosclerotic lesions and vascular injury was significantly attenuated[34]. It also has been reported that AGE/RAGE is implicated in diabetic related macrovascular complications, arterial injury, as well as the progression of diabetic nephropathy and retinopathy[36]. In a T2DM leptin receptor deficient (*db*/*db*) mouse model, using RAGE blocking antibody, left ventricular diastolic chamber stiffness and the cardiac systolic function was attenuated in conjunction with reduced fibrosis. It has been proposed the multiple outcomes of AGE/RAGE signaling operate through protein kinase C (PKC). Utilizing cell culture experiments to model T1DM and T2DM hyperglycemic growth conditions *in vitro*, PKC activity was increased and followed by subsequent activation of various prostaglandins, cytokines, and increased ECM protein expression[22]. Immunoblotting experiments using of cellular lysates revealed PKC-α, -β Ⅰ, -β Ⅱ, -δ, -ε, and -ζ isoform activity was increased in endothelial cells[37].

The PKC kinase family is defined based upon their second messenger requirements. The conventional PKC family, which includes PKC-α, -β Ⅰ, -β Ⅱ, and -γ, is stimulated by calcium, phosphatidylserine, diacylglycerol, or phorbol-12-myristate-13-acetate. Members of the novel PKC group, which includes -δ, -ε, -θ and -η are also activated by the above ligands with the exception of calcium. The atypical PKC family, which includes -ζ and -ι/λ, cannot be activated by any of the above second messengers[38]. To date, PKC isoform activation has been associated with vascular alterations, including increased permeability, contractility, ECM synthesis, cell growth, and apoptosis[37], and these perturbations in vascular cell homeostasis have been shown to be mediated by differing PKC isoforms[37]. Of these isoforms, PKC-β and PKC-ζ emerged as a preferred substrate in the aortic and cardiac tissue of diabetic mice[39,40]. Additional examination of multiple PKC isoforms has identified of PKC-ζ as the most plausible target for RAGE phosphorylation[41].

PKC-ζ is involved in propagating a multiple of cascade pathways that lead to mitogen-activated protein kinase (MAPK) activation. The MAPK family plays a pivotal role in numerous cellular processes, including development, phenotype differentiation, and ECM protein synthesis. In a study by Koya *et al*[37], ERKs were demonstrated to be activated in a PKC-dependent manner. ERKs are a subfamily of MAPKs involved in signaling cascades responsible for multiple cellular functions, such as differentiation and proliferation. Stimulation of ERK signaling cascades involve activation of a molecular switch, Raf, to trigger a stepwise serine kinase cascade through activation of Raf, MAPK kinase kinase, MAPK kinase, MAPK, and ERK[42]. Activated ERK will translocate into the nucleus to activate transcription factors to initiate cellular proliferation, differentiation, and matrix accumulation[43-45].

AGE/RAGE and PKC-ζ signaling cascades have been demonstrated to increase ERK activation, both independently as well as synergistically; thereby PKC-ζ serves as a common molecular mediator between these two different cascades[46,47]. Phosphorylation of RAGE at Ser391 is a ligand-dependent mechanism that is required to perpetuate AGE/RAGE signaling[41]. PKC-ζ has been demonstrated to phosphorylate Ser391 of the intracellular RAGE domain. However in order for this to occur, PKC-ζ must be activated by Ras, a small GTPase, to initiate the cascade[41]. Recently, our lab and others have found that Rap1a, a small Ras-like GTPase, may also play a role in AGE/RAGE signaling in diabetes.

RAP1A: A MOLECULAR SWITCH

Rap1a, member of the Ras superfamily, operates as a binary molecular switch. This relay system is capable of transmitting a number of diverse signals from members of the Ras superfamily to effect changes in nuclear transcription, thus coupling extracellular stimulation to intracellular signaling cascades. In fact, Rap1a has been demonstrated to participate in hypertrophic pathways, integrin-mediated adhesion, cell attachment, migration, and cell junction formation. Studies have shown that Rap1a induced-ERK1/2 activation contributes to vascular pathologies as well as plays a role in the cardiovascular ion channels responsible for rhythmic heart function[48].

Rap1a utilizes a guanine nucleotide exchange factors (GEFs), that causes the dissociation of a bound GDP allowing for a new GTP molecule to bind. GTPase-activating proteins (GAPs) will then hydrolyze the newly

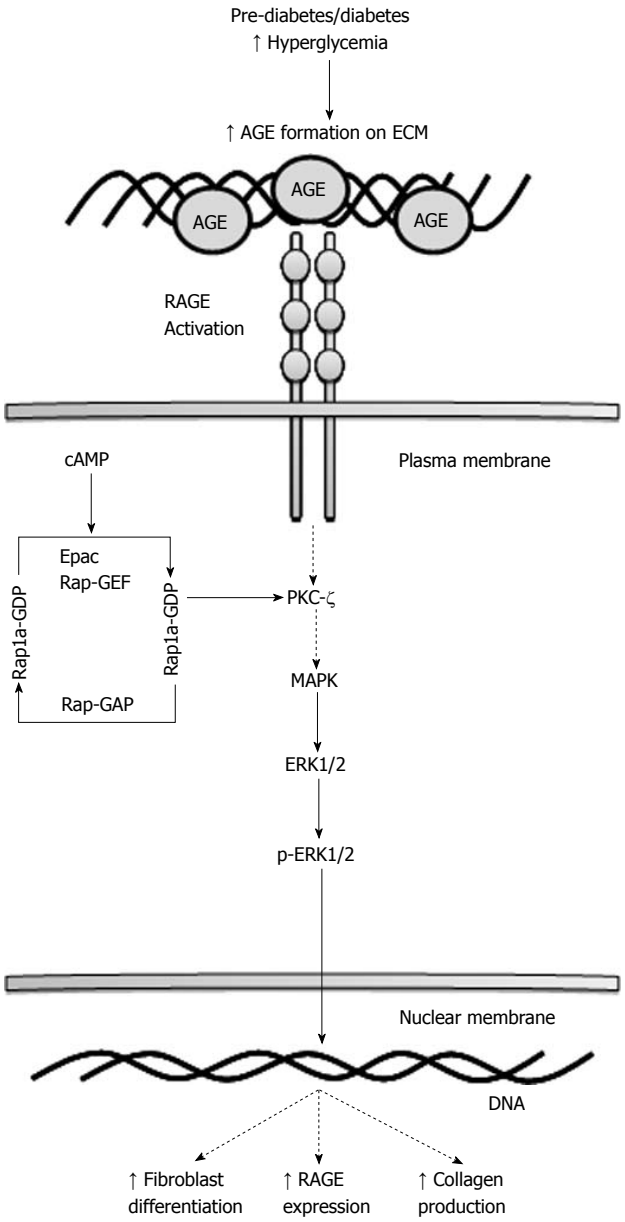

Figure 1 Rap1a in advanced glycation end product/the receptor for advanced glycation end product signaling. A potential role for Rap1a as a molecular switch mediating the AGE/RAGE signaling pathway in type 2 diabetes mellitus. Increased Rap1a activity may stimulate PKC-ζ to further promote matrix accumulation, RAGE expression and fibroblast differentiation to myofibroblasts. AGE: Advanced glycation end product; RAGE: The receptor for AGE; ECM: Extracellular matrix; cAMP: Cyclic AMP; GAP: GTPase-activating protein; PKC-ζ: The ζ isotype of protein kinase C; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

bound GTP to GDP forcing the cycle to run in one direction. In this capacity, Rap1a rotates between the inactive GDP-bound and the active GTP-bound substrate. In addition, Rap1a has been demonstrated to be activated by at three second messengers, specifically cyclic AMP (cAMP), calcium, and diacylglycerol[49]. It is now recognized that a number of GEFs can be directly activated by cAMP whereby cAMP binding causes a conformational change in the GEF permitting nucleotide exchange. Of particular interest are the GEFs known to activate Rap1a. These are commonly referred to as cAMP-GEF or more specifically Epac (Exchange Protein directly Activated by cAMP). Epac proteins have been demonstrated to bind cAMP and activate Rap1a GTPases[50]. Conversely, Rap1a-GAP will hydrolyze GTP at the asparagine side chain, thereby rendering Rap1a inactive.

The dynamic control of Rap1a activation has been shown to be facilitated by protein kinase A (PKA) and Epac through cAMP-dependent cascades[51]. Both PKA and Epac proteins contain a cAMP binding domain and are sensitive to fluctuations to mediate Rap1a activation[48]. While PKA can phosphorylate the C-terminus of Rap1a, PKA-mediated activation is not necessary for cAMP stimulation of Rap1 by Epac. In fact, there have been extensive studies that have established Epac's involvement in various cAMP-related cellular functions, such as cellular adhesion, that were previously attributed to PKA[52,53]. These cAMP sensitive proteins may act independently, synergistically, or possible antagonistically depending upon cellular distribution, concentration, and location to regulate Rap1a-mediated cellular functions. Our understanding of the Rap1a pathway is centered on the biological responses elicited by PKA-dependent pathways triggering downstream ERK1/2 activation[30]. However, recent studies have suggested a PKA-independent pathway for Epac-Rap1a activation of downstream signaling effectors[54]. Precise investigation of the discrete role and involvement of Rap1a is necessary within a number of signaling model systems.

AGE/RAGE and Rap1a-induced ECM accumulation in diabetes

To date, there is paucity in the literature describing the interactions between Rap1a and the AGE/RAGE signal pathway in T2DM. Early studies described Rap as being up-regulated in multiple organs of diabetic rats[55]. Of note, these studies also demonstrated that diacylglycerol can activate a Rap/Raf/MAPK-mediated signal cascade through PKC, however no specific PKC isoform was identified[55]. Furthermore, in a study by Panchatcharam *et al*[56], increased Rap1 expression was reported in smooth muscle cells under hyperglycemic conditions, yet no distinction between Rap1a or Rap1b subtypes was made. Taken together, there is evidence that Rap1a under hyperglycemic conditions will increase downstream kinase activity *via* ERK1/2 activation, and these events would ultimately influence other signaling pathways, including the AGE/RAGE cascade, to promote ECM accumulation to contribute to cardiac complications in diabetic patients.

Both the AGE/RAGE signaling cascade and Rap1a utilize and activate similar signaling pathways, such as ERK1/2 MAPK, NF-κB and JNK, which are involved in cell growth, ECM synthesis and myofibroblasts differentiation. It has been demonstrated that fibroblasts treated with transforming growth factor-β, a known fibrosis mediator, myofibroblasts differentiation and ECM deposition is increased[17,57]. Furthermore, studies by Yan *et al*[57], showed that major molecular mediators, like ERK1/2

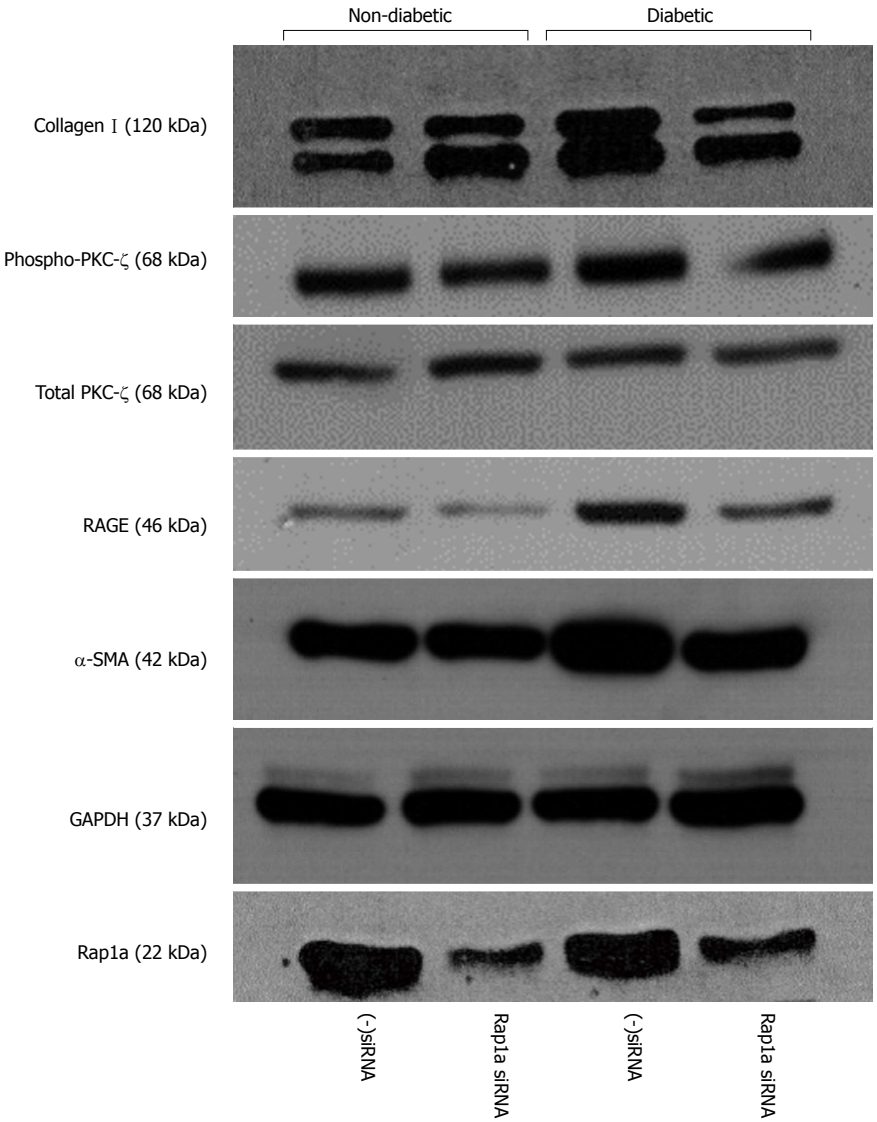

Figure 2 siRNA Rap1a knockdown in diabetic cardiac fibroblasts. Cardiac fibroblasts were isolated from age-matched 16 wk-old *db*/*wt* (non-diabetic) and *db*/*db* (diabetic) mice and using siRNA targeted to Rap1a silenced transcription and translation of Rap1a resulting in noticeable decreases not only in Rap1a expression, but also the downstream signaling outcomes RAGE, collagen I , phospho-PKC-ζ and α-SMA protein expression. RAGE: The receptor for the advanced glycation end product; PKC-ζ: The ζ isotype of protein kinase C; α-SMA: α-smooth muscle actin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

MAPK, involved in fibroblast growth factor-2 mediated angiogenesis were down regulated when Rap1a was depleted. Lastly, Jeyaraj *et al*[48] implicated Rap1a in roles that were intimately associated with the ECM remodeling process. Taken together, Rap1a and AGE/RAGE have been demonstrated to associate with increased myofibroblast formation and interstitial fibrosis independently. Figure 1 illustrates Rap1a's potential role in mediating the AGE/RAGE signaling pathway as discussed in the context of this review. While there is some evidence of a functional interplay between AGE/RAGE and Rap1a, the exact molecular interactions have not been fully characterized.

A series of studies by our laboratory suggest that Rap1a plays a role in fibrosis and myofibroblast differentiation in isolated diabetic and non-diabetic fibroblasts. Silencing Rap1a mRNA in diabetic fibroblasts returned profibrotic markers to nondiabetic levels. Isolated cardiac fibroblasts from 16 wk-old non-diabetic (heterozygous, wt/db) and diabetic (homozygous, *db*/*db*) mice were treated with siRNA targeted to Rap1a and a negative control of scrambled siRNA (data not shown) was used. 48-h post siRNA treatment, noticeable decreases were measured, not only in Rap1a expression, but also RAGE, collagen I , phospho-PKC-ζ, and α-smooth muscle actin protein expression (Figure 2). Inhibiting Rap1a protein expression down-regulated the molecular switch used to activate PKC-ζ to promote AGE/RAGE-mediated fibrosis. While these studies are still in a preliminary stage, we are working to expand our understanding of the significance of these alterations using not only siRNA technology, but also generating a double knockout mouse model to ascertain the role Rap1a plays in diabetic cardiomyopathy.

CONCLUSION

From the evidence that is presented, a cellular and mo-

lecular mechanism for Rap1a-mediated activation of AGE/RAGE-dependent myocardial remodeling exists. This review is the first of its kind to provide Rap1a as a unique target for therapeutic strategies aimed at reducing chronic hyperglycemia-mediated ECM production and accumulation in diabetic patients. While much still needs to be performed to increase our understanding of this causal relationship, our laboratory is working towards defining the signaling cascade involving Rap1a and PKA in the AGE/RAGE signaling cascade which ultimately mediates fibroblast myocardial remodeling. These studies provide insight into the inter-signaling components of this cascade that could ultimately help in reducing ECM production and accumulation during hyperglycemia in T2DM patients.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Donna M Gordon, Neeta Kumari, Rebecca A Worsham and Carter G Holland for their assistance in preparing this manuscript.

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P- Reviewer: Georgescu A, Kusmic C **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Cardiac adipose tissue and its relationship to diabetes mellitus and cardiovascular disease

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Received: May 29, 2014 Revised: September 23, 2014
Accepted: October 14, 2014
Published online: December 15, 2014

Abstract

Type-2 diabetes mellitus (T2DM) plays a central role in the development of cardiovascular disease (CVD). However, its relationship to epicardial adipose tissue (EAT) and pericardial adipose tissue (PAT) in particular is important in the pathophysiology of coronary artery disease. Owing to its close proximity to the heart and coronary vasculature, EAT exerts a direct metabolic impact by secreting proinflammatory adipokines and free fatty acids, which promote CVD locally. In this review, we have discussed the relationship between T2DM and cardiac fat deposits, particularly EAT and PAT, which together exert a big impact on the cardiovascular health.

Key words: Epicardial adipose tissue; Pericardial adipose tissue; Type 2 diabetes; Cardiovascular disease

Core tip: Diabetes, a cardiovascular disease equivalent, has considerable effects on the cardiovascular system. Its impact works systemically, but may have more association with epicardial and pericardial adipose tissue locally at the level of the heart. These cardiac tissues have great interplay with diabetic patients and have potential to influence cardiovascular disease.

Original sources: Noyes AM, Dua K, Devadoss R, Chhabra L. Cardiac adipose tissue and its relationship to diabetes mellitus and cardiovascular disease. *World J Diabetes* 2014; 5(6): 868-876 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/868.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.868

INTRODUCTION

More than 25 million United States adults have type-2 diabetes mellitus (T2DM) and this figure will likely reach 50 million by 2050[1,2]. The relationship between metabolic diseases such as T2DM and regional fat deposits, particularly epicardial adipose tissue (EAT) and pericardial adipose tissue (PAT), play an important role in the development of cardiovascular diseases (CVD). Both EAT and PAT are a subset of visceral adipose tissue (VAT) associated with T2DM. They are metabolically active visceral fat deposits found around the heart[3], that are strongly associated with CVD including coronary artery disease (CAD) and the development of cardiac arrhythmias, predominantly due to the secretion of pro-inflammatory mediators and cytokines[4]. In this paper, we review the emerging evidence of impact of T2DM on VAT and the specific role of EAT and PAT both as a cardiac risk marker and as a potentially active player in the development of cardiovascular pathology.

RESEARCH

We searched MEDLINE and PubMed for original articles published between 1984 and 2014, focusing on epicardial adipose tissue and type 2 diabetes mellitus. The search terms we used, alone or in combination, were

"epicardial fat", "epicardial adipose tissue", "pericardial fat", "pericardial adipose tissue", "insulin resistance", "type 2 diabetes mellitus", "metabolic syndrome", "cardiovascular disease", "coronary artery disease", "congestive heart failure", and "atrial fibrillation", which yielded 121 articles. All articles identified were English-language, full-text papers and abstracts. We finally selected 87 articles, which were relevant to our current discussion.

T2DM AND CARDIAC VISCERAL FAT

Cardiac disease is the leading cause of death in T2DM, and many have sought to determine the mechanism of development of cardiac dysfunction[5]. Interestingly, diabetic patients with no evidence of CAD or hypertension have also been found with cardiac abnormalities, even when they are asymptomatic. Studies have shown that the metabolic derangements in T2DM primarily contribute to the cardiac problems[6], which, in part, are due to increase in visceral fat deposits and being frequently accompanied by disorders of glucose metabolism[7]. Obesity, specifically abdominal VAT, is an independent risk factor for CVD[8], and is prominent in patients with T2DM[7]. Moreover, studies have shown the correlation between excessive adipose tissue deposition and development of diabetes[9]. Central and VAT is associated with endocrine disorders due to the release of substances such as free fatty acids (FFA), leptin, adiponectin, pro-inflammatory agents, and decreased anti-inflammatory factors. As a result, it often results in unfavorable glucose metabolism and T2DM[10,11]. It has also been well demonstrated that pre-diabetic and diabetic patients are associated with significantly higher PAT burden compared to normoglycemic patients[12]. In a cross sectional study, the impact of obesity and T2DM on adipocytokines (adiponectin, leptin and resistin), inflammatory markers [tumor necrosis factor-α (TNF-α), Interleukin (IL)-6 and high sensitive C-reactive protein (HsCRP)] were evaluated[13]. Obesity was found to significantly lower adiponectin levels, while increasing leptin and IL-6 levels along with HsCRP. There is also a strong association between the increased expression of resistin, another adipocyte-secreted factor, and insulin resistance[14], with the burden of EAT volume being greater in individuals with metabolic syndrome, increased insulin resistance and diabetes mellitus[15,16], and is significantly higher in patients with T2DM than in non-diabetic subjects[4]. The serum profile of coronary artery bypass grafting patients showed significantly higher levels of HsCRP and lower levels of adiponectin compared to body mass index (BMI)-matched controls, supporting the role of VAT in causation of systemic inflammation[17]. Adiponectin has been shown to have a protective role with anti-inflammatory properties suppressing TNF-α and IL-6[13,18]. Hypoadiponectin levels in obesity along with elevated TNF-α, HsCRP and IL-6 were shown to correlate with insulin resistance seen in this population[13]. Interestingly leptin and resistin levels were not shown to consistently correlate with insulin resistance.

EAT and omental fat were shown to have broadly comparable pathogenic mRNA profile[17]. EAT and PAT are both forms of VAT, which store lipids and have demonstrated increased expression of the above mentioned hormones, chemokines and cytokines, with the addition of monocyte chemotactic protein-1 and IL-1β[19]. These adipokines also impair insulin-signaling pathways leading to insulin resistance and reduced nitric oxide (NO) synthesis, causing unopposed vasoconstriction[20]. Thus, the endocrine function of EAT and PAT play a significant role in patients with metabolic syndrome. In fact, the examination of EAT and PAT found that PAT is associated with VAT and metabolic syndrome features such as T2DM, than that of EAT[21]. On the other hand, EAT thickness showed independent positive correlation with metabolic parameters including postprandial glucose ($P = 0.049$), HbA1c level ($P < 0.001$), and homeostasis model assess of insulin resistance ($P = 0.047$)[22]. EAT accumulation was seen to strongly correlate with serum fibroblast growth factor 21, which is known to improve insulin sensitivity despite an increment in its serum levels in T2DM patients. Thus, excessive EAT in T2DM patients may exert bivalent, unfavorable and adaptive effects on progression of cardiovascular diseases[23].

In obese patients with T2DM, adipocytes from epicardial fat infiltrate the myocardium, which refers to a strong association of intra-myocardial fat content to the echocardiographic epicardial fat thickness. Similarly, EAT has been found to be significantly related to intra-abdominal visceral fat, suggested by echocardiographic studies[24,25], and PAT may increase up to 400 g in T2DM patients (with 100 g in healthy lean people)[26]. Yang *et al*[12] demonstrated the burden of PAT in diabetic and pre-diabetic subjects, revealing that PAT volume was much higher in pre-diabetics and diabetics as compared to normoglycemic subjects.

However, it is important to distinguish EAT and PAT from obesity-specific lipotoxic cardiomyopathy, in which excessive fat proliferates inside cardiac muscle causing left ventricular remodeling and eventually cardiomyopathy. This develops after subcutaneous adipose tissues and VAT are unable to accommodate the excess fat in the obese patients leading to intracellular accumulation of lipids and FFA, eventually forming myocardial steatosis[27].

ANATOMICAL, METABOLIC AND FUNCTIONAL DIFFERENCES BETWEEN EAT AND PAT

Epicardial and pericardial adipose tissue are close, however anatomically clearly different. EAT is not symmetrically distributed around the heart (Figure 1). EAT volume and thickness varies depending on the location (Figure 2). PAT (Figure 3) has a different embryonic origin than that of EAT as it originates from the embryonic primitive thoracic mesenchyme[24], and clinically are different. In

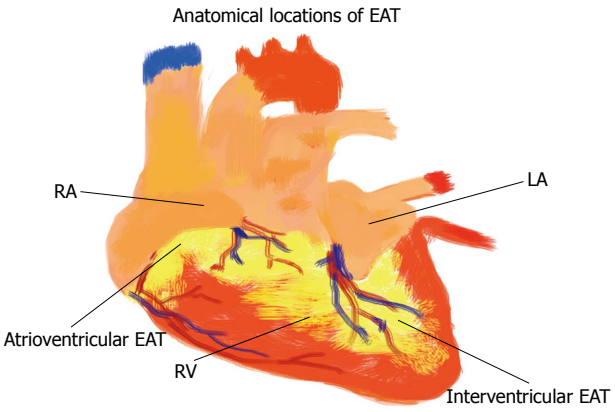

Figure 1 Anatomical locations of epicardial adipose tissue. RV: Right ventricle; RA: Right atrium; LA: Left atrium; EAT: Epicardial adipose tissue (yellow color refers to EAT).

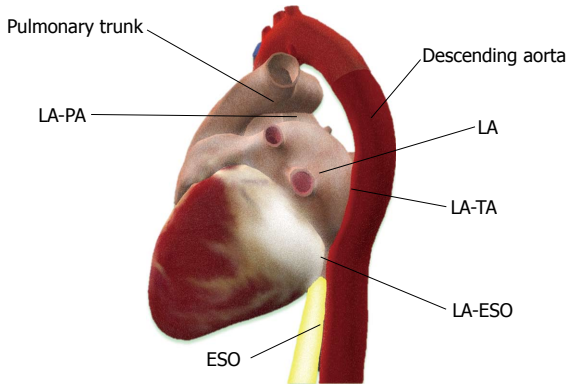

Figure 2 Periatrial epicardial adipose tissue around left atrium (heart in lateral axis view). LA-PA: Epicardial adipose tissue (EAT) between left atrium and pulmonary artery; LA-TA: EAT between left atrium and thoracic aorta; LA-ESO: EAT between left atrium and esophagus.

the existing literature, the terminologies have often been erroneously overlapped without clear differentiation between these two entities. Some suggest the use of a terminology, which encompasses three types of fat around the heart: epicardial, pericardial and paracardial fats. In this terminology, paracardial fat often refers to the fat located on the external surface of the parietal pericardium, while the term pericardial fat is used to represent EAT plus paracardial fat. It is important to be familiar with these terms to avoid confusion. In our opinion, it is rather more important to differentiate the "true pericardial fat" from "paracardial fat" as these two have different endocrine and metabolic properties. The true pericardial fat (epi-pericardial fat) should encompass the epicardial and pericardial fat (*i.e.*, fat located above the myocardium and up to the parietal pericardium; epicardial fat being located between the outer wall of the myocardium and the visceral layer of pericardium and pericardial fat being located between the visceral and the parietal pericardium), while paracardial fat should clearly be considered as the fat located outside the parietal pericardium.

EAT is a metabolically active visceral fat deposit found around the heart, between the pericardium and

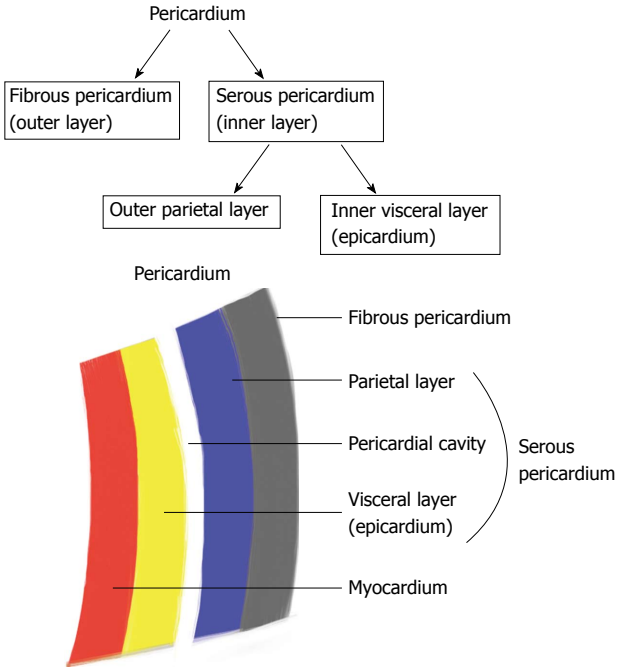

Figure 3 Pericardium/Pericardial layers.

myocardium[3]. EAT can be found in highest concentration in the atrioventricular and interventricular grooves and alongside the coronary arteries, and lesser so around the atria, over the free wall of the right ventricle and over the apex of the left ventricle. PAT may be defined as EAT plus paracardial fat, whereas paracardial fat is located on the external surface of the parietal pericardium within the mediastinum[28]. EAT varies from PAT and other local fat depots in the size of its adipocytes, where as epicardial adipocytes are smaller in size and high in number (high number of pre-adipocytes). The best imaging tool for quantification of both EAT and PAT remains uncertain. Their thicknesses and volumes can be evaluated by echocardiography, computed tomography (CT) or magnetic resonance imaging (MRI)[24,29]. Due to distinct attenuation values of fat on chest or cardiac CT and MRI, EAT and PAT are both readily identified with ability to calculate the tissue volume and thickness. Furthermore, MRI accurately correlates with EAT and PAT seen on echocardiography imaging[30].

Biochemically, EAT and PAT are different. Investigation into EAT and PAT suggests that these two tissues have different metabolic and physiologic properties[31]. Under physiological situations, EAT is cardioprotective which can be explained by its anti-atherogenic/anti-inflammatory properties, high FFA release and uptake and low glucose requirements, serving as a major source of energy to the heart and thermoregulatory properties[32]. It is also known to provide mechanical support to the coronary arteries as well as anti-toxic effects by protecting heart from high levels of FFA. In diabetics, lack of insulin impairs cardiac glucose transport and oxidation, resulting in FFA becoming the preferred means of energy supply[33]. To make available this increased requirement

Table 1 Studies showing the relationship between pericardial adipose tissue and epicardial adipose tissue and the development of coronary artery disease

Ref.	Year	Diagnostic modality	Results
Taguchi *et al*[86]	2001	Computerized tomogram	Pericardial fat was the strongest independent variable for severity of CAD, determined by coronary angiogram
Jeong *et al*[41]	2007	Echocardiogram	Epicardial fat thickness significantly correlated with the severity of CAD in patients with known CAD
Ahn *et al*[38]	2008	Echocardiogram	Epicardial adipose tissue was an independent predictor of CAD
Greif *et al*[36]	2009	Computerized tomogram	Patient with any coronary plaque showed a significantly higher pericardial adipose tissue volume compared to patients without coronary plaques
Shemirani *et al*[40]	2012	Echocardiogram	Confirms the presence of association between epicardial fat thickness and severity of CAD

CAD: Coronary artery disease.

of the heart for FFA, the diabetic heart upregulates its luminal lipoprotein lipase (LPL) activity, which can result in abnormal FFA supply and utilization by the heart tissue, potentially initiating cardiac dysfunction[33]. Importantly, EAT has low levels of LPL and acetyl-CoA as compared to subcutaneous fat[34], though the cardio-protective role of PAT is not clear[31]. Despite these protective qualities, EAT in excess can become cardio-toxic resulting in local inflammatory changes and cardiac dysfunction[32,35]. In non-diabetic patients with excessive EAT, the presence of fatty acid binding protein-4 in epicardial adipocytes, and its increased expression, promotes the development of metabolic syndrome[32] and T2DM.

CARDIAC ADIPOSITY, DIABETES MELLITUS AND CAD

PAT and EAT have firmly been recognized as a contributor to the development of CAD[36-41], and several cross sectional studies (Table 1) have shown similar results. PAT is emerging as a novel risk factor for CVD development[42] and progression[43], as CAD has been shown to correlate with PAT more consistently than other general measures of adiposity like body mass index or waist circumference[42]. PAT volume has been a predictor of increased death and disability for CVD[44], and independently linked with coronary artery calcification (CAC)[45]. EAT has also been shown to correlate with CAC[43] and has a statistically significant correlation between EAT and CAC in both diabetic and non-diabetic patients ($P = 0.01$, $r = 0.60$; $P = 0.02$, $r = 0.38$, respectively)[46]. The Multi-Ethnic Study of Atherosclerosis study showed a stronger correlation between PAT and the incidence of future coronary heart events in a group of patients without history of CAD, than that of other cardiac risk factors such as BMI or waist circumference[42].

EAT has been studied more extensively than PAT. EAT differs from PAT, not only in its location, but also by its blood supply. EAT derives its blood supply from coronary circulation, whereas PAT is supplied by non-coronary sources[32]. There is a functional and anatomic relationship between EAT and muscular components of the heart as these components share the same coronary blood supply, due to the lack of fascia separating the adipose tissue and myocardial layers[3]. Because of the highly metabolic paracrine and endocrine functions of EAT, it has been proposed to play a role in the pathogenesis of CVD by contributing to increased carotid intima media thickness (CIMT) in those with metabolic syndrome[47], CAD[37-41], increased left ventricle (LV) mass[48] and diastolic dysfunction[49,50]. The release of pro-inflammatory and pro-atherogenic factors into the circulation advancing CVD is more significantly linked to VAT accumulation, metabolic syndrome and other situations related to oxidative stress[32]. Pathophysiological effects of abnormal EAT may be explained by the expression of an enzyme-sPLA2-IIA which is generally found in human atherosclerotic lesions[32]. In patients with CAD, catalase levels in EAT are lower than in subcutaneous fat resulting in higher oxidative stress, which further contributes to atherosclerosis.

It is the close anatomical relationship between EAT and the coronary arteries, combined with its biologically active properties that participates in the pathogenesis of diabetic coronary atherosclerosis[4,51]. Iacobellis *et al*[52] demonstrated that the expression of anti-inflammatory and antiatherogenic properties of adiponectin was approximately 40% lower in the EAT of patients with CAD than in that of normal controls.

Apart from above, EAT was also shown to play an important role in the prediction of no-reflow phenomenon in ST elevation myocardial infarction treated with primary percutaneous intervention (PCI)[53]. The no-reflow was defined as < 70% ST-segment resolution following primary PCI. EAT has also been shown to be one of the independent factors associated with restenosis post-stenting warranting target vessel revascularization[54]. Smooth muscle proliferation, secondary to the local inflammatory mediators, have been postulated as mechanism of restenosis in this population[54].

EAT volume also has a significant role in promoting CVD and was shown to be positively and independently related to coronary atherosclerotic burden[55], and was significantly increased in patients with acute coronary syndrome[14]. Multivariate logistic regression analysis indicated that EAT thickness was an independent indicator for significant coronary artery stenosis after adjusting for traditional risk factors (OR = 1.403, $P = 0.026$)[22]

assessed by cardiovascular magnetic resonance imaging in asymptomatic T2DM patients. Echocardiographic measurement of EAT thickness ⩾ 7 mm was shown to identify individuals with higher probability of coronary atherosclerosis[56]. Furthermore, EAT thickness ⩾ 5 mm in general population may identify individuals with higher likelihood of detectable carotid atherosclerosis, but did not have any significant association with CIMT[57]. However, EAT thickness in patients with metabolic syndrome showed a linear positive correlation with CIMT[47]. Similar association was also found in human immunodeficiency virus receiving highly active antiretroviral therapy[58]. These studies establish that the correlation between EAT and CIMT is stronger in high-risk individuals prone to atherosclerosis than in the general population. It also demonstrates the existence of independent paracrine effects in addition to the endocrine effect, to account for the consistent association of EAT and coronary atherosclerosis[59].

CARDIAC ADIPOSITY AND VENTRICULAR FUNCTION

EAT and associated inflammatory cytokines, particularly hypoadiponectin levels and reduced NO synthesis, may have direct effect on myocardium causing dysfunction independent of ischemic pathophysiology[60]. PAT was shown to be significantly associated with LV diastolic dysfunction in people with CAD and normal ejection fraction independent of other risk factors including diabetes and hypertension[61]. Variation in regional fat distribution has been reported in patients on peritoneal dialysis[62]. Increased EAT thickness determined by echocardiogram in such patients was shown to be the most powerful determinant of LV diastolic dysfunction among other variables[63]. In addition to the paracrine metabolic effect as discussed earlier, mechanical effect of increased PAT has also been shown to contribute to the pathophysiology of diastolic dysfunction[63]. Additionally, patients with LV diastolic dysfunction had significantly increased EAT volumes[64].

On contrary, in patients with congestive heart failure (CHF) and severely reduced left ventricular ejection fraction (LVEF), EAT has been found to be significantly reduced[65]. LV function in such patients correlated best with EAT/Left Ventricular Remodeling Index ratio[65], raising a possible protective role of EAT to remodeling myocardium. Khawaja *et al*[66] demonstrated similar results with a stepwise decrease in EAT volume from controls to patients with moderate CHF (LVEF 35%-55%) and severe heart failure (LVEF < 35%). Though the paracrine metabolic effects and possible role as source of FFA to myocardium in demand has been postulated as mechanism for this correlation[65], the exact pathophysiology remains elusive. Further study is needed to access the possible confounding role of lipid lowering therapies to this finding in such patients.

CARDIAC ADIPOSITY, DIABETES MELLITUS AND ARRHYTHMOGENICITY

Obesity is a well-established risk factor for atrial fibrillation (AF), as altered atrial electrical function is considered an important mechanism for the relation of obesity and increased AF risk. Atrial tissue in diabetic subjects demonstrates persistent oxidative stress compared with nondiabetics; which can potentially play a role in the development of interatrial conduction delay[67]. Evidence on the impact of EAT thickness, particularly in the area of posterior left atrium, is associated with persistent AF[68,69]. PAT is also associated with a higher incidence of AF, both paroxysmal (OR = 1.11, 95%CI: 1.01-1.23, P = 0.04) and persistent (OR = 1.18, 95%CI: 1.05-1.33, P = 0.004), independent of other risk factors[69]. PAT's unique anatomic proximity to the myocardium and atrial conduction system may modify atrial electrophysiology and promote subsequent risk for arrhythmogenesis[70]. Based on PAT's influence on altered P-wave indices (PWI), potential mechanisms by which increases in PAT may lead to changes in atrial conduction include prolonged atrial depolarization, diminished voltage, and heterogeneous atrial activation related to fibrosis, hypertrophy, and fatty myocardial infiltration[70].

Two independent studies reported significant association of pericardial fat volume with AF both paroxysmal and persistent even after adjustment for traditional risk factors[69,71]. The possible mechanisms speculated were secondary to increase in left atrial size associated with pericardial fat[72,73] and local inflammatory effects induced by pericardial adipose tissue as discussed earlier *via* paracrine and endocrine route. This speculation was based on the evidence that systemic inflammation marked by CRP was associated with presence of AF and also predicted the patients at risk for future development of AF[74].

PWI and PAT were found to be associated independent of ectopic visceral and intra-thoracic fat depots[70], supporting the role of PAT in atrial conduction. Voltage-dependent PWI (P-Wave amplitude, P wave area and P wave terminal force) may be enhanced by hypertrophy of left atrium seen with pericardial fat. At the same time it may also be decreased due to fibrosis and effects on summation vector secondary to insulation effect[70]. The insulation effect does not affect the voltage-independent PWI (P wave duration and PR interval), however hypertrophy and fibrosis may still affect the conduction time[70]. P-wave terminal force is more closely associated with pericardial fat than other voltage-dependent PWI[70]. This is due to the fact that blocked posterior inter-atrial bundles seen with PAT causes anterior to posterior activation of left atrium resulting in a terminal negative deflection on the electrocardiogram in lead V1. PAT has been questioned to contribute to the P wave dispersion seen in obese individuals[71].

With further advancements in imaging, thickness of the posterior peri-atrial fat pad between left atrium

and the esophagus was found to correlate with the AF burden[68]. Their proximity to the pulmonary vein ostia would explain the correlation, as triggers for AF initiation are located in the pulmonary vein ostia[75]. EAT total and inter-atrial septal thickness was shown to be related to left atrial volume independently even after adjustment for other confounding factors[76]. PAT has also been associated with increased risk of AF recurrence after ablation[77]. PAT volume has also been identified as a novel risk factor for post-operative AF after coronary artery bypass grafting[78].

MANAGEMENT OF EAT AND PAT

As excessive cardiac adipose tissue have correlations with poor cardiovascular outcomes, research into possible reversal of the tissue has been studied. Weight loss through bariatric surgery and calorie restriction has shown a corresponding decrease in EAT volume and thickness. EAT thickness decreased in obese subjects who underwent an aggressive 6-mo long weight loss program (mean 20 kg) by adhering to a very low-calorie diet (900 kcal/d)[79]. Similarly, weight loss after bariatric surgery (average weight loss of 40 kg) was associated with a decrease in EAT thickness[80]. Conversely, the compared effects of pioglitazone and metformin treatment in T2DM patients demonstrated an increase in PAT volume in pioglitazone-treated patients after 24 wk[81]. Nonetheless, the correlation between increased cardiac adipose tissue has been associated with several features of metabolic syndrome, including fasting insulin[82]. Further studies are needed to show the effects of controlling these measures with changes in size of the cardiac adipose tissues.

CONCLUSION

Cardiac adipose tissue is metabolically active and associated with various metabolic derangements in the body leading to insulin resistance, atherosclerosis, metabolic syndrome and CVD. It has become clear that the adipose tissue around the heart is a critical indicator of CVD burden. Lifestyle and medical improvements may reduce this impact, as the evidence through the use of ultrasound has documented that weight loss is associated with a decrease in pericardial fat stores in both non-diabetic[79,83,84] and diabetic[85] subjects. In diabetics, metabolic derangements are significantly linked with cardiac adiposity, thus it should be considered screening for EAT or PAT as CVD risk factors in diabetic patients. Many aspects between EAT and PAT overlap. Clinicians and researchers must have a clear understanding of their physiological and pathological differences to expand on screening, managing and reducing the impact that EAT and PAT have on CVD.

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P- Reviewer: Gillessen A, Saeki K, Tziomalos K
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Risk factors for mortality in children with diabetic keto acidosis from developing countries

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Received: July 3, 2014 Revised: August 15, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

Diabetic keto acidosis (DKA) is the major cause for mortality in children with Diabetes mellitus (DM). With increasing incidence of type 1 DM worldwide, there is an absolute increase of DM among children between 0-14 year age group and overall incidence among less than 30 years remain the same. This shift towards younger age group is more of concern especially in developing countries where mortality in DKA is alarmingly high. Prior to the era of insulin, DKA was associated with 100% mortality and subsequently mortality rates have come down and is now, 0.15%-0.31% in developed countries. However the scenario in developing countries like India, Pakistan, and Bangladesh are very different and mortality is still high in children with DKA. Prospective studies on DKA in children are lacking in developing countries. Literature on DKA related mortality are based on retrospective studies and are very recent from countries like India, Pakistan and Bangladesh. There exists an urgent need to understand the differences between developed and developing countries with respect to mortality rates and factors associated with increased mortality in children with DKA. Higher mortality rates, increased incidence of cerebral edema, sepsis, shock and renal failure have been identified among DKA in children from developing countries. Root cause for all these complications and increased mortality in DKA could be delayed diagnosis in children from developing countries. This necessitates creating awareness among parents, public and physicians by health education to identify symptoms of DM/DKA in children, in order to decrease mortality in DKA. Based on past experience in Parma, Italy it is possible to prevent occurrence of DKA both in new onset DM and in children with established DM, by simple interventions to increase awareness among public and physicians.

Key words: Diabetic keto acidosis; Mortality; Cerebral edema; Sepsis; Shock; Delayed diagnosis

Core tip: Mortality in Diabetic keto acidosis (DKA) among children from developed countries is due to cerebral edema and is very low. The mortality in DKA among children from developing countries is due to higher incidence of cerebral edema, sepsis, shock and renal failure. Delayed diagnosis is the root cause for high mortality in children with DKA from developed countries. There is an urgent need to increase the awareness about diabetes among the public and physicians.

Original sources: Poovazhagi V. Risk factors for mortality in children with diabetic keto acidosis from developing countries. *World J Diabetes* 2014; 5(6): 932-938 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/932.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.932

INTRODUCTION

Diabetes mellitus in children is on the rise for past few decades. On an average 78000 children are diagnosed with diabetes every year[1]. One among every five children with newly diagnosed type 1 diabetes mellitus (DM) is

found to be an Indian[1]. In this world pandemic of diabetes with efforts to control type 2 DM it is easy that the needs of type 1 DM who are only 10% of people with diabetes is forgotten. Occurrence of type 1 DM is on the rise among children between 0 and 14 years of age. Majority of children present with diabetic keto acidosis (DKA) at onset and this rate is inversely proportional to prevalence of DM in the population[2]. Death in DM is predominantly due to DKA. Mortality rates in developed countries and developing countries show much variation. Similarly the cause for mortality in DKA varies between developed countries and developing countries. Cerebral edema is the predominant cause for mortality in children with DKA from developed countries, while recent data from developing countries has shown higher incidence of cerebral edema, sepsis, shock and renal failure as the cause for death in DKA[3]. Delayed diagnosis has been identified as a major risk factor associated with mortality in children from Chennai-India[3].

Overall mortality in children with DKA varies from 0.15% to 0.35% in developed countries like Canada, United States and United Kingdom[4-7] and from 3.4% to 13.4% in developing countries like India, Pakistan and Bangladesh[8-14]. Cerebral edema is the major cause for mortality in DKA[15,16]. Occurrence of cerebral edema varies from 0% to 5.5% in developed countries[17-19] and is reported to vary from 24%-26% in developing countries[10]. Literature on reasons for such high mortality and associated factors for death in children with DKA in developing countries are very recent and majority of these are based on retrospective studies. Whether factors associated with mortality are pre hospital in nature or treatment related needs to be understood. In the editorial published in Indian Pediatrics during the year 2004, titled "What determines the outcome of DKA in children from a developing country?" author has raised issues regarding fluid therapy in DKA[20]. The role of amount and rate of fluid administration in the management of DKA associated cerebral edema is still controversial. Traditionally cerebral edema has been linked to fluid therapy in DKA. A recent article titled 'Warning from India' has addressed the issue of high mortality and high incidence of sepsis and cerebral edema in children with DKA from a developing country[21]. Association of sepsis may have a great impact on fluid therapy in DKA.

DKA RELATED CEREBRAL EDEMA

Cerebral edema has been the major risk factor for mortality in children with DKA world over. Despite decades of management of DKA the exact cause for cerebral edema in DKA is yet to be understood. Whether hypo perfusion related ischemia leading to cytotoxic edema or reperfusion induced vasogenic edema, is the cause for cerebral edema is controversial. However initial cytotoxic edema followed by subsequent vasogenic edema can very well contribute to development of cerebral edema in DKA. Also the role of inflammatory mediator release, glucotoxicity, uremia or acidosis in causing cerebral edema, is not clearly understood. Occurrence of cerebral edema can be at the time of presentation or during therapy up to initial 24 h. Predisposing factors for cerebral edema in children with DKA have been identified in various studies in developed countries. Identified factors are disease related or treatment related or both. Identified factors vary from young age at presentation, new onset disease, rate and amount of fluids used for resuscitation, blood urea nitrogen, body mass index, initial osmolality, rapid fall in osmolality, failure of sodium to rise with treatment, use of bicarbonate for correction of acidosis, insulin infusion in the first hour of therapy of DKA or bolus insulin therapy in DKA[22-30]. There has been no consistency among the factors identified for occurrence of cerebral edema in various studies published till date.

Occurrence of cerebral edema from developing countries has been found to be as high as 26% among a cohort of children admitted at a pediatric intensive care unit in north India[11]. Literature on reasons for such high incidence of cerebral edema from developing countries is very scarce. Studies by Tiwari *et al*[11] from Chandigarh-India have identified fluid refractory shock, higher volume of fluids at admission and respiratory failure requiring ventilation to be significant risk factors for cerebral edema in DKA. However only fluid refractory shock, azotemia and younger age were identified to be significant risk factors for cerebral edema in multivariate analysis[11]. Literature from Chennai-India has revealed cerebral edema in 24% of study group[3]. In this prospective study of 118 children with DKA, specific risk factor related mortality for cerebral edema was 43%. A higher fluid bolus at the emergency room for resuscitation was a significant therapy related factor for cerebral edema by univariate analysis. Cerebral edema was significantly associated with altered sensorium, lower $PaCO_2$ at admission, delayed diagnosis and failure of sodium to rise with therapy by multivariate analysis. Both the studies from India have identified higher fluids as risk factors for cerebral edema in univariate analysis but were not significant in multivariate analysis[3,11]. This may be an important observation in developing countries where sepsis has been an important factor associated with increased mortality in children with DKA. Too much of fluid for resuscitation resulting in cerebral edema is still controversial in DKA. Similarly less fluid in a child with DKA and shock may also worsen risk of cerebral edema and renal failure. Sepsis by itself will demand large volumes of fluid boluses in a child. Hence recommendations regarding fluid therapy based on guidelines from developed countries where sepsis and shock are not major factors in children with DKA needs to be addressed for future guidelines when applied to developing countries. Whether there is a need for more liberal fluid therapy in DKA in developing countries where sepsis, shock and renal failure have been identified to be risk factors for mortality needs to be addressed by multicentric trials.

SEPSIS IN DKA

Sepsis in DKA as a risk factor for increased mortality has been identified in studies form developing countries like India, Pakistan and Bangladesh[8-12,14]. Majority of these studies were based on retrospective data. Still they have identified sepsis as a definite risk factor for mortality in children with DKA. Though infections have not been identified to be a major comorbid state in children with type 1 diabetes from developed countries, studies from Chennai-India has shown that infections are much more common in children with diabetes in comparison to children without diabetes[31]. In this context infections do play a major role in children with DKA. Sepsis not only precipitates DKA, also complicates fluid therapy, predisposes to renal failure and is associated with increased mortality in DKA based on data from developing countries. Jayashree *et al*[10] in 2004 from India published their retrospective study in DKA. They reported that among 64 children with DKA 30 children had foci of infection. Respiratory infection in 10, soft tissue infection in 10, meningitis in 3, hepatitis in 2, peritonitis, chronic suppurative otitis media, tonsillitis, ethmoiditis and oral and vulval candidiasis in one each. Cerebral edema and complicating sepsis were reported to result in poor outcome in children with DKA. In their series, sepsis was the triggering factor in one third of cases. In study from Chennai-India, infections were encountered in 61 children among the study group of 118 children[3]. Of these 49 had identified focus of infection (41.5%). Culture positive sepsis was seen in 12% of children with DKA and is associated with specific risk related mortality of 57%. Other infections encountered were pneumonia, urinary tract infections, skin and soft tissue infections, mucormycosis, acute suppurative otitis media, enteric fever and peritonitis. Kanwal *et al*[12] from Delhi India has identified 32.7% of study group (18 of the 55 children) to have sepsis. Documented infection were reported to be 16.3%. Urinary tract infection, pneumonia, diaorrhea and culture positive sepsis were the identified infections. Study by Tiwari *et al*[11] published in 2012 had revealed 58% of study population as sepsis as per standard definition. However only 1/5th of this group had a focus of infection identified. Respiratory tract was the focus in 6, gastrointestinal in 4, sinonasal mucormycosis, urinary tract infection (UTI), acute otitis media, peritonitis, tonsillitis and cellulitis one each. Infections have been reported in 48% of children with DKA by Zabeen *et al*[14] from Bangladesh. Mortality in their study group were attributed to cerebral edema and sepsis. Respiratory infections were commonest followed by urinary tract infections, sepsis and pneumonia.

Studies from Iran by Asl *et al*[32] reported that among 63 children with DKA 13 of them had infections. This was inclusive of pneumonia, tuberculosis, diarrhea and upper respiratory infections. The study documented acute renal failure in 4.7%. Clinical diagnosis of sepsis as well as shock may be over diagnosed in children with DKA. Presence of fever in DKA signifies infection and the focus need to be identified. The criteria for systemic inflammatory response (SIRS), when applied to children with DKA may lead to over diagnosis of sepsis. Tachycardia and tachypnea as criteria can be explained by dehydration and keto acidosis rather than sepsis and lactic acidosis. Lactic acidosis in DKA could be due to sepsis, hypovolemia or due to disturbed carbohydrate metabolism perse. Similarly DKA is known to be associated with leucocytosis and this is not specific for sepsis in DKA[33]. Leukocytosis is a part of stress response in DKA and may be seen in up to 50%-60% of children with DKA[34]. One needs to be very cautious about diagnosing sepsis based on the criteria for SIRS in DKA. Any child with fever or a focus of infection along with any of the above criteria can be taken as sepsis complicating DKA. Current guidelines from developed countries where sepsis is not a major factor, do not recommend antibiotics in DKA. Based on literature evidence from developing countries, sepsis is more common and sepsis complicating DKA has increased mortality. Hence antibiotics may be empirically considered in children with fever or refractory shock despite the absence of obvious focus of sepsis, until infections have been ruled out in DKA among children from developing countries.

SHOCK IN DKA

Shock as a presentation in DKA is rare in literature from developing countries[35]. International Society for Pediatric and Adolescent Diabetes clinical practice consensus guidelines 2009 compendium states the following "Despite of their dehydration, patients continue to maintain normal blood pressure and have considerable urine output until extreme volume depletion and shock occurs, leading to a critical decrease in renal blood flow and glomerular filtration"[36]. However it is uniformly reported in literature from developing countries that shock at presentation in children with DKA is fairly common. Studies from Pakistan[8] have revealed incidence of shock to be 19.3% in their study and overall mortality was 3.4%. Tiwari *et al*[11] from Chandigarh, India documented in their study that 48% of study population with DKA at the pediatric intensive care unit had hypotensive shock at presentation and of them 30% needed inotropes. Kanwal *et al*[12] from India have documented in their study on 55 DKA children, incidence of shock to be 18.1%, 10.9% were due to hypovolemia and 7.25% were due to septic shock. Study from Chennai[3] has shown occurrence of shock at presentation in DKA to be 12% and specific risk factor related mortality in DKA to be 53%. According to another study from Chennai, India among the 23 children with DKA 10 presented with shock[37]. However criteria used to assess shock in those children and severity of shock had not been discussed. Shock in DKA is a combination of hypovolemia and sepsis. To differentiate between the two is difficult and most of the time it may be a combination of hypovolemia and sepsis. The clinical evidence for hypovolemia in DKA is not reliable

as published in literature. Intra cellular dehydration in DKA may not be clinically evident and hence degree of dehydration may be under diagnosed. Capillary refill time in DKA cannot be relied as a sign of shock in DKA[38]. Tachycardia could be a physiological response to dehydration in DKA and this needs caution while interpreting it as a sign of shock. Tachypnea for similar reasons is due to acidosis which is predominantly keto acids and cannot be interpreted as a sole evidence of hypo perfusion and lactic acidosis. Altered sensorium in DKA can be explained by cerebral edema, severe acidosis or shock in DKA. This feature cannot be relied as a sign of poor end organ perfusion of shock. In developing countries where cerebral edema, shock, sepsis and renal failure are reported to be common in DKA, diagnosis based on clinical features alone may be challenging for the pediatrician at the emergency department. Hence the criteria for septic shock or hypovolemic shock may need to be applied with clinical judgment in children with DKA. Presence of fever, hypotension, wide pulse pressure in septic shock, clinical evidence of dehydration in hypovolemia may be better indicators of type of shock in DKA. Similarly is the assessment of dehydration in shock. Clinical signs of dehydration may not be evident in DKA. Since initial dehydration is predominantly intra cellular there may not be obvious clinical evidence of dehydration at presentation in a child with DKA. This might lead to underestimation of degree of dehydration in DKA. With recent literature from developing countries regarding shock and sepsis in DKA, we need to reappraise the existing guidelines from developed countries for fluid therapy in children with DKA. Whether less fluid is harmful or more fluid is harmful needs to be answered by well planned fluid trials for children with DKA from developing countries.

RENAL FAILURE IN DKA

Renal failure in children with DKA is a complication unheard of in literature from developed countries[39]. Children from developing countries presenting with renal failure in DKA is not uncommon. Studies from Iran by Asl *et al*[32] reports that 4.7% of children with DKA had acute renal failure. Studies from Bangladesh by Zabeen *et al*[14] have shown the incidence of renal failure to be 3.7% in DKA. Published literature from Chennai, India[40] revealed acute renal failure in DKA to be 11.5%. Mortality among children with DKA and acute renal failure was documented to be 40%-72%. Sepsis, shock and rhabdomyolysis causing acute renal failure have been reported in the series. Renal failure leads to difficulty in diagnosis as well as management of DKA. Oliguria and anuria as criteria for renal failure is not reliable in DKA due to osmotic diuresis of hyperglycemia. Similarly, urea and creatinine values may be elevated in DKA due to prerenal causes like dehydration which declines with adequate fluids. Subsequent elevation in creatinine cannot be taken as a definite criterion for renal failure as the commonly used calorimetric method of creatinine estimation is likely to be associated with spurious elevation due to interference by ketones. Fluid restriction in a child with sepsis and shock (hypovolemic or septic) for fear of cerebral edema during management of DKA may predispose to renal failure. Child with severe dehydration with delay in diagnosis may present with acute tubular necrosis leading to renal failure in DKA. Management of renal failure in DKA poses great difficulty for the treating physicians. Following needs to be considered in renal failure in DKA-modification of amount and type of fluids for therapy, consideration of using bicarbonate, varied metabolism and sensitivity of insulin. Peritoneal dialysis in such children also leads to severe fluctuations of blood glucose levels. Presently there are no standard guidelines for management of renal failure in DKA among children. There is an urgent need for such guidelines based on the existing evidence from developing countries.

DELAYED DIAGNOSIS OF DKA

What predisposes children with DKA to such complications in developing countries needs to be addressed urgently. Delayed diagnosis in DKA has been identified to be one of the factors for mortality in DKA in studies from Chennai-India[3] and also recently has been presented as an e poster at a conference, from Chandigarh-India[41]. Children with diabetes presenting with DKA at the onset has been attributed to delay in diagnosis in developed countries. Missed diagnosis of DKA predisposing the child to DKA is common in literature. However delay in diagnosis as a significant risk factor for mortality in DKA has been identified only from India[3]. This study reported that children with DKA had 1-5 physician visits prior to diagnosis of DKA. Children with DKA were more likely to have consulted a physician prior to diagnosis of DKA as reported in literature from developed countries. Rosenbloom[39] from US had mentioned that children with new onset DKA has been seen in physician's office prior to diagnosis without adequate history and laboratory evaluation. In infants and young children symptoms may be nonspecific and this needs a high index of suspicion to diagnose DKA. Literature reports that DKA has been misdiagnosed as surgical emergencies with acute abdomen[42]. Bui *et al*[43] from Canada published that among 285 children with DKA, 38.8% and 1104 children with diabetes with no DKA, 34.4% had at least one medical visit during the week before diagnosis (p-026). Ali *et al*[44] had published in 2011 that 30% of newly diagnosed children have had at least one related medical visit prior to diagnosis, suggesting the condition is being missed by doctors. Majaliwa *et al*[45] from Africa mention in their article that DKA can easily be misdiagnosed as cerebral malaria or meningitis in busy emergency reception areas of most hospitals in Africa. Literature reveals similar studies from Tunisia and Tanzania[46,47]. However none of these studies have identified delayed diagnosis as a risk factor for mortality in children with DKA. Study from Chen-

Table 1 Reasons for delayed diagnosis in diabetic keto acidosis

Reason
Lack of parental awareness about diabetic symptoms
Lack of awareness among physicians
Mis-interpretation of diabetic symptoms
Exclusive treatment of intercurrent illness only
Lack of finger prick estimation of blood glucose
Non recognition of lab abnormalities
Lack of immediate referral
Delay in transport to appropriate center
Improper referral
Due to parental causes (native treatment, social reasons and economic constraints)

nai[3] has identified delayed diagnosis in DKA in 64.8% of children with new onset DKA. Eighty-four point seven percent of infants and 58% of school children with DKA had delayed diagnosis. Delayed diagnosis was encountered in 12 of 13 children who died of DKA in their study. Specific risk factor related mortality for delayed diagnosis was 21% in the study[3]. Factors identified for the delayed diagnosis in their study is summarized in Table 1. Tachypnea in DKA has been universally misdiagnosed as bronchopneumonia, bronchiolitis or acute severe asthma in children. Polyuria and polydipsia have been misinterpreted as UTI in most of the children. Abdominal pain is misinterpreted as worm infestation and acute gastritis. Dehydration in the presence of vomiting is usually treated as acute gastroenteritis. Recent onset bed wetting is seen as a sign of stress in school children. Literature reveals up to 15%-86% of children with DKA not been diagnosed as diabetic at the first physician consultation[43,44,48,49].

Delay due to missed diagnosis is universal in DKA among children from developed and developing countries. Literature also reveals that, simple estimation of blood glucose by capillary method using finger prick has been used very rarely in physician consultation room[3]. A simple investigation in the physician's consultation room could have led to the diagnosis in children who had their diagnosis missed prior to DKA. Similarly, laboratories did not alert the treating physician or the parent when they measured high blood glucose or documented glycosuria in children[3]. Inappropriate referral was again reason for delay in specific management as the facilities for management of DKA by a structured diabetic care team or intensive care pediatrician is not universally available in developing countries. All treating physicians should have access to the standard treatment protocols for management of children with DKA or should have an access to help through hot line facilities at times of need. These factors coupled with lack of knowledge about emergency free public transport facilities, economic constraints and unhealthy cultural practices lead to delay in management of DKA in children from developing countries.

CONCLUSION

Analyzing the magnitude of problems in DKA in children from developing countries it is obviously evident that the mortality rates and reasons for such high mortality in DKA to be very different from developed countries. However majority of standard treatment protocols followed in developing countries are based on recommendation from developed countries. Root cause for majority of these complications could be delayed diagnosis of DKA. There is an urgent need for modified protocols for children with DKA and shock or renal failure. Fluid trials in such children is an urgent need of the hour. There exists difficulty in recognizing the symptoms of diabetes among parents and physicians[50]. With regard to delay in diagnosis there is a need for creating awareness among parents and physicians regarding clinical features of DM and DKA. The best strategy would be to identify DKA early and refer them to appropriate centers for management immediately. The laboratories should raise high risk alert immediately to the parent or the physician when they encounter a child's report with hyperglycemia and/or glycosuria.

As majority of the risk factors identified for mortality in DKA among children from developing countries are pretreatment factors, the ultimate aim of future programmes should be to prevent DKA in children. DKA occurring in new onset DM or in a known diabetic child is considered as a preventable health care failure. Vanelli *et al*[51,52] in Parma, Italy have proved that simple awareness programmes in schools and physicians office in the form of posters depicting signs of diabetes have helped over 5 years to reduce occurrence of DKA to zero. This has been proved to be successful even years after the programme was stopped. Studies from Australia have shown reduction in the rate of DKA at initial diagnosis of diabetes, during awareness campaigns[53]. Similar models with modification to local needs can help prevent delay in diagnosis of DKA among children from developing countries. Initiatives and awareness programmes need to be implemented in countries like India where the magnitude of the problem is likely to increase over years. It is time that emergency interventions are undertaken to minimize deaths in DKA in developing countries. Increased awareness among parents, school teachers and physicians is urgently warranted for early diagnosis and prevention of mortality in DKA. Creating awareness through nationwide diabetic awareness day can help an earlier diagnosis of DKA among children.

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P- Reviewer: Hsu YH **S- Editor**: Gong XM
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Clinical therapeutic strategies for early stage of diabetic kidney disease

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Author contributions: All authors contributed to this work.
Supported by A Grant from Novo Nordisk Pharma, to Kitada M; A Grant-in-Aid for Scientific Research (C) to Kitada M, No. 24591218; A Grant for Promoted Research from Kanazawa Medical University to Kitada M, No. S2012-4; A Grant-in-Aid for Scientific Research (B) to Koya D, No. 25282028; A Grant-in-Aid for Challenging Exploratory Research to Koya D, No. 25670414; A grant for Specially Promoted Research from Kanazawa Medical University to Koya D, No. SR2012-06; and the 4th Annual Research Award Grant of the Japanese Society of Anti-Aging Medicine, to Koya D
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Received: November 26, 2013 Revised: March 8, 2014
Accepted: April 17, 2014
Published online: June 15, 2014

Abstract

Diabetic kidney disease (DKD) is the most common cause of chronic kidney disease, leading to end-stage renal disease and cardiovascular disease. The overall number of patients with DKD will continue to increase in parallel with the increasing global pandemic of type 2 diabetes. Based on landmark clinical trials, DKD has become preventable by controlling conventional factors, including hyperglycemia and hypertension, with multifactorial therapy; however, the remaining risk of DKD progression is still high. In this review, we show the importance of targeting remission/regression of microalbuminuria in type 2 diabetic patients, which may protect against the progression of DKD and cardiovascular events. To achieve remission/regression of microalbuminuria, several steps are important, including the early detection of microalbuminuria with continuous screening, targeting HbA1c < 7.0% for glucose control, the use of renin angiotensin system inhibitors to control blood pressure, the use of statins or fibrates to control dyslipidemia, and multifactorial treatment. Reducing microalbuminuria is therefore an important therapeutic goal, and the absence of microalbuminuria could be a pivotal biomarker of therapeutic success in diabetic patients. Other therapies, including vitamin D receptor activation, uric acid-lowering drugs, and incretin-related drugs, may also be promising for the prevention of DKD progression.

Key words: Diabetic kidney disease; Glycemic control; Renin-angiotensin system inhibitor; Multifactorial therapy; Remission and regression of albuminuria

Core tip: We show the significance of targeting the remission/regression of microalbuminuria in type 2 diabetic patients, leading to protection against the progression of diabetic kidney disease (DKD) and cardiovascular events. To achieve the remission/regression of microalbuminuria, the multifactorial intervention and the early detection of microalbuminuria with continuous screening is important, as management of DKD. Multifactorial intervention includes glucose, blood pressure and lipid control. Additionally, other therapies, including vitamin D receptor activation, uric acid-lowering medicine and incretin-related medicines may be promising for preventing the progression of DKD. We review the current standard treatment for DKD and other prospective therapies for DKD.

Original sources: Kitada M, Kanasaki K, Koya D. Clinical therapeutic strategies for early stage of diabetic kidney disease. *World J Diabetes* 2014; 5(3): 342-356 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/342.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.342

INTRODUCTION

The prevalence of diabetes mellitus is increasing. According to the International Diabetes Federation Atlas of 2012, the estimated diabetes prevalence in 2012 was 371 million, representing 8.3% of the world's adult population; it was predicted that by 2030, the number of people with diabetes in the world will have risen to 552 million[1]. Long-term diabetes results in vascular changes and dysfunction, and diabetic complications are the major causes of morbidity and mortality in diabetic patients. Among diabetic vascular complications, diabetic kidney disease (DKD) is a common cause of chronic kidney disease (CKD) and is a leading cause of end-stage renal disease (ESRD)[2]. In addition, microalbuminuria/proteinuria and a decline in the glomerular filtration rate (GFR) are observed in CKD and are recognized as independent risk factors for the development of ESRD and the onset of cardiovascular diseases, respectively. Therefore, it is important to establish therapeutic strategies for DKD.

The pathogenesis of DKD is complex and has not yet been completely elucidated. Hyperglycemia is one major factor that is responsible for the pathogenesis of DKD[3]. Moreover, elevated systemic blood pressure and intra-glomerular pressure, which are associated with the renin-angiotensin system (RAS), several cytokines and growth factors induced by metabolic and hemodynamic factors, and abnormal lipid metabolism are involved in the pathogenesis of DKD[4,5]. Current therapeutic strategies targeting these mechanisms, particularly the control of blood glucose and blood pressure, have been established in many hallmark clinical trials. In addition, a reduction in microalbuminuria is more frequent than progression to overt proteinuria, and a multifactorial control approach is important for this reduction in microalbuminuria, leading to reductions in renal and cardiovascular risk. In this review, we discuss the current standard treatment and other prospective therapies in DKD (especially early stage) that target a reduction of albuminuria.

MECHANISMS OF ALBUMINURIA IN DKD

Albuminuria is a signature feature of DKD. Albuminuria in DKD is predominantly due to impairment in the glomerular filtration barrier, consisting of the glomerular endothelial cells, the glomerular basement membrane (GBM), and the podocytes[6]. Podocytes are the predominant component of this barrier, and the reduced number of podocytes due to increased apoptosis and detachment from the GBM is observed in the diabetic kidney, resulting in leakage of albumin through areas of denuded podocytes[7-12]. In addition to a decrease in podocyte number and density, the widening of the foot processes, shortening of the slit diaphragm/loss of slit diaphragm proteins, changes in the actin cytoskeleton, and decreases in negative charge may cause albuminuria in DKD[13-15]. Furthermore, endothelial cell injuries in diabetic conditions leading to reduced nitric oxide production[16,17], altered vascular endothelial growth factor (VEGF) signaling[18,19] and diminished glycocalyx[20] also play pivotal roles in albuminuria. Glomerular endothelial cells and podocytes crosstalk through several mediators, including VEGF-A[19], angiopoietin-1[21,22] and -2[23] and activated protein C[24]; therefore, the missing link between endothelial cells and podocytes in diabetic conditions contributes to dysfunction of both cell types, resulting in increased albuminuria[25]. Glomerular hemodynamic changes, including hyperfiltration and hyperperfusion, are observed in diabetic conditions and hypertension. Elevated intraglomerular pressure creates a shear stress on the glomeruli and leads to an increase in albuminuria due to endothelial and podocyte dysfunction[26]. Vascular endothelial dysfunction is closely related to the pathogenesis of the initiation of cardiovascular disease (CVD); albuminuria also reflects glomerular endothelial dysfunction. Therefore, albuminuria is a marker of both glomerular and early systemic endothelial dysfunction[27,28].

Tubular cell injury may also contribute to albuminuria by impairing proximal tubular albumin and protein reabsorption. In diabetes, proximal tubular reuptake of albumin and protein may be impaired by high glucose[29], transforming growth factor (TGF)-β[30], or angiotensin Ⅱ [31]. Tubulointerstitial injury is enhanced and the ability to reabsorb albumin and protein is further reduced, along with the development of glomerular disease, and there is a direct correlation between the degree of tubulointerstitial scarring and the extent of albuminuria[32].

SCREENING METHODS AND DIAGNOSIS OF DIABETIC KIDNEY DISEASE

The early clinical sign of DKD is elevated urinary albumin excretion, referred to as microalbuminuria, which progresses to overt proteinuria and leads to nephritic-range proteinuria in some cases. Increasing albuminuria (proteinuria) leads to a decline in renal function, which is defined in terms of the GFR[33] and generally progresses inexorably to ESRD 6-8 years after the detection of overt proteinuria[34]. Microalbuminuria is defined as a urinary albumin-creatinine ratio (ACR) of 30-299 mg/g creatinine (Cr), and macroalbuminuria is defined as an ACR > 300 mg/g Cr[35]. Elevated ACR should be confirmed in the absence of urinary tract infection in two additional first-void specimens collected during the following 3 to 6 mo[35].

Microalbuminuria in diabetic patients has been recognized as a useful biomarker for diagnosing DKD and as a predictive factor for progression to ESRD. In most patients with diabetes, CKD should be attributed to diabetes if any of the following is true: macroalbuminuria is present, microalbuminuria is present in the presence of diabetic retinopathy, or type 1 diabetes has occurred with a duration of at least 10 years[35]. However, other causes of CKD should be considered in the presence of any of the following circumstances: diabetic retinopathy is absent, GFR is low or rapidly decreasing, proteinuria is increasing or there is evidence of nephritic syndrome,

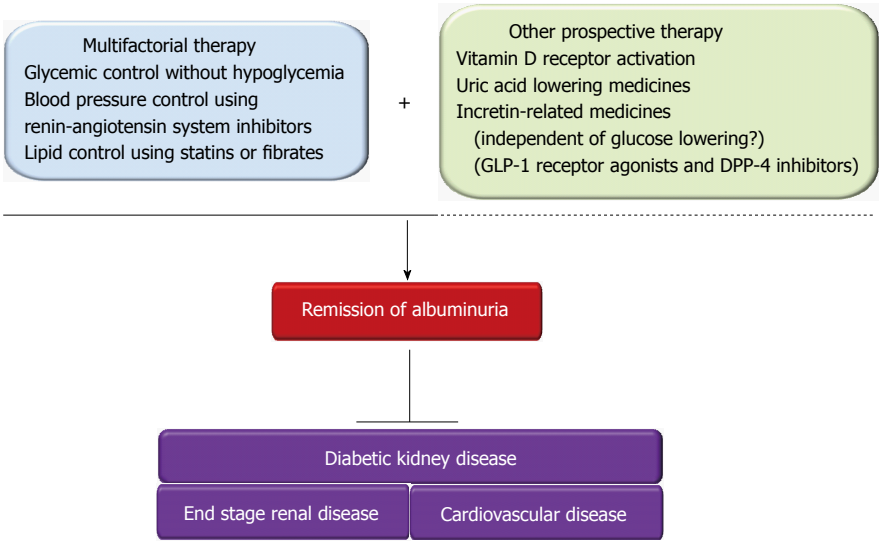

Figure 1 Therapeutic strategy for diabetic kidney disease. Multifactorial therapy, consisting of glycemic, blood pressure, and lipid control, is recommended to prevent the progression of diabetic kidney disease (DKD). The remission and regression of albuminuria by multifactorial therapy may be closely associated with reduced risk of progression of both DKD and cardiovascular disease. In addition to these therapies, vitamin D receptor activation, uric acid-lowering drugs, and incretin-related drugs should be considered in the prospective treatment of DKD.

refractory hypertension is noted, active urinary sediments are present, signs or symptoms of other systemic diseases are present, or a > 30% reduction in GFR has occurred within 2-3 mo after initiation of treatment with an angiotensin converting enzyme (ACE) inhibitor or angiotensin Ⅱ receptor blocker (ARB)[35].

Additionally, microalbuminuria has been shown to be closely associated with an increased risk of cardiovascular morbidity and mortality[36-38]. In a sub-analysis of the United Kingdom Prospective Diabetes Study (UKPDS), the cardiovascular mortality of type 2 diabetic patients with microalbuminuria was reported to be two times higher than that of patients with normoalbuminuria[39]. Therefore, microalbuminuria is not only a biomarker for the diagnosis of DKD but is also an important therapeutic target for improving the prognosis of renal and cardiovascular risk in diabetic patients.

THERAPEUTIC STRATEGY FOR DIABETIC KIDNEY DISEASE

The current therapeutic strategy for DKD is shown in Figure 1. A multifactorial therapeutic approach, including glycemic control, blood pressure management, and lipid control, is recommended to prevent the progression of DKD. The remission and regression of albuminuria as a result of multifactorial therapy may be closely associated with reduced risk of both the progression of DKD and cardiovascular disease. In addition to these therapies, vitamin D receptor activation, uric acid-lowering drugs, and incretin-related drugs are potential treatments for DKD.

BLOOD GLUCOSE CONTROL

Targeting HbA1c

Chronic hyperglycemia is the main causal factor underlying diabetic vascular complications, including DKD. Multiple potential molecular mechanisms have been proposed to explain hyperglycemia-induced diabetic complications. Some of the most-studied mechanisms include disruption of the polyol pathway, activation of the diacylglycerol-protein kinase C pathway, increased oxidative stress, increased formation and activity of advanced glycation end products, and activation of the hexosamine pathway[3]. Additionally, alterations in signal transduction pathways induced by hyperglycemia or toxic metabolites have been reported to cause multiple vascular dysfunctions, such as abnormal blood flow, and increased apoptosis, inflammation, and accumulation of extracellular matrix in the kidney by alteration of gene expression or protein function[3]. Therefore, glycemic control is fundamentally necessary to prevent the onset and progression of DKD by influencing both hyperglycemia itself and hyperglycemia-induced metabolic abnormalities; this premise has been supported by several randomized controlled clinical trials in both type 1 and type 2 diabetes, as described below.

Type 1 diabetes: In the Diabetes Control and Complications Trial (DCCT), the average HbA1c levels were 7% and 9% for the intensive and conventional therapy groups, respectively. Intensive glycemic control was associated with a risk reduction of 34% for the onset of microalbuminuria and a risk reduction of 56% for progression to overt albuminuria[40]. Additionally, in the Epidemiology of Diabetes Interventions and Complications study (the follow-up study to the DCCT), intensive glycemic control prevented the onset of microalbuminuria (yielding a decrease in the odds ratio of 84% for the intensive therapy group) and the progression to overt albuminuria (yielding a decrease in the odds ratio of 59% for the intensive therapy group) at 7-8 years after the end of the DCCT, although the differences in HbA1c

Table 1 Effects of intensive glucose control on the onset and progression of diabetic kidney disease

Study	HbA1c		Outcome of albuminuria or renal events
	Intensive treatment	Conventional treatment	
ACCORD[45]	6.4% *vs* 7.6%		21% ↓ in onset of microalbuminuria 32% ↓ in progression to macroalbuminuria
ADVANCE[46]	6.5% *vs* 7.3%		9% ↓ in onset of microalbuminuria 30% ↓ in progression to macroalbuminuria 21% ↓ in renal events New onset macroalbuminuria Doubling of serum Cr Kidney replacement therapy Death due to kidney disease
VADT[47]	6.9% *vs* 8.4%		32% ↓ in progression from normal to microalbuminuria or macroalbuminuria 37% ↓ in progression from normal to microalbuminuria to macroalbuminuria 34% ↓ in any increase in albuminuria

ACCORD: Action to Control Cardiovascular Risk in Diabetes; ADVANCE: Action in Diabetes and Vascular disease: Preterax and Diamicron MR Controlled Evaluation; VADT: Veterans Affairs Diabetes Trial.

between the intensive and conventional therapy groups had decreased over that time. Moreover, 24 cases exhibited elevated serum Cr levels (≥ 2.0 mg/dL); of these 24 cases, 19 were in the conventional therapy group, and five were in the intensive therapy group[41]. In the follow-up study conducted 22 years after initiation of the DCCT[42], a decrease in the GFR (< 60 mL/min per 1.73 m^2) was observed in the intensive therapy group, with a risk reduction of 50% compared with the conventional therapy group. The decrease in GFR per year was significantly suppressed in the intensive therapy group compared with the conventional therapy group (intensive therapy: conventional therapy, 1.27 mL/min per 1.73 m^2/year: 1.56 mL/min per 1.73 m^2/year).

Type 2 diabetes: In the UKPDS33, the median HbA1c levels were 7.0% and 7.9% for the intensive and conventional therapy groups, respectively. The development of diabetic microvascular complications, including nephropathy, in the intensive therapy group was reduced by 25% relative to the conventional therapy group[43]. In the follow-up study conducted 10 years after the end of the UKPDS, the development of microvascular complications, including nephropathy, in the intensive therapy group was still reduced by 24% compared with the conventional therapy group, although the differences in the HbA1c levels between the intensive and conventional therapy groups had diminished.

In the Kumamoto Study, the average HbA1c levels were 7.5% and 9.8% for the intensive and conventional therapy groups, respectively. The cumulative rates for the development and progression of nephropathy after 6 years were 7.7% for the intensive therapy group and 28.0% for the conventional therapy group in the primary prevention cohort; these rates were 11.5% and 32.0%, respectively, in the secondary intervention cohort. In this study, an HbA1c < 6.9% was identified as the target for preventing the onset and progression of diabetic nephropathy[44]. In the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, the HbA1c levels at the end of the study were 6.4% and 7.5% for the intensive and conventional therapy groups, respectively. Intensive glycemic control reduced the onset of microalbuminuria by 21% and the progression to macroalbuminuria by 32%[45] (Table 1). In the Action in Diabetes and Vascular disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) study, the HbA1c levels at the end of the study were 6.5% and 7.3% for the intensive and conventional therapy groups, respectively. Intensive glycemic control resulted in a 21% reduction in new onset or worsening nephropathy defined by new onset macroalbuminuria, doubling of serum Cr, need for kidney replacement therapy, or death due to kidney disease. Additionally, intensive glycemic control decreased the development of new onset microalbuminuria by 9%, and development of macroalbuminuria by 30%[46] (Table 1). In the Veterans Affairs Diabetes Trial (VADT) study, the HbA1c levels at the end of the study were 6.9% and 8.4% for the intensive and conventional therapy groups, respectively. Intensive glycemic control resulted in a 32% reduction in the progression from normal albuminuria to microalbuminuria or macroalbuminuria, and a 37% reduction in the progression from normal albuminuria to microalbuminuria to macroalbuminuria, and a 34% reduction in any increase in albuminuria[47] (Table 1). The ACCORD, ADVANCE, and VADT studies showed the beneficial effects of intensive glycemic control on the prevention of microalbuminuria and reduced progression to macroalbuminuria; however, these studies showed no significant benefit of more intensive glycemic control on Cr-based estimates of GFR (eGFR).

Based on the results from these clinical trials, the Standards of Medical Care in Diabetes 2014 of the American Diabetes Association (ADA)[33], the Kidney Disease Improving Global Outcomes (KDIGO) 2012 Clinical Practice Guidelines for the Evaluation and Management of Chronic Kidney Disease and the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines for the management of diabetes with CKD[35] recommend a target HbA1c < 7.0%

Table 2 Target of blood pressure in diabetic kidney disease (different of clinical guidelines)

Clinical guideline	Target population	Target of blood pressure
Standard of Medical Care in Diabetes-2014 (ADA)	Diabetic patients	< 140/80 mmHg (< 130 mmHg, younger patients if it can be achieved without undue treatment burden)
KDIGO 2012 CKD guideline	Diabetes + CKD	
	UAE < 30 mg/24 h or ACR < 30 mg/gCr	≤ 140/90 mmHg
	UAE ≥ 30 mg/24 h or ACR ≥ 30 mg/gCr	≤ 130/80 mmHg
JNC8	Diabetic patients	< 140/90 mmHg
	CKD patients	

CKD: Chronic kidney disease; UAE: Urinary albumin excretion; ACR: Albumin creatinine ratio; ADA: American Diabetes Association; KDIGO: The kidney Disease Improving Global Outcomes; JNC8: The Eighth Joint National Committee.

to prevent or delay the progression of DKD. However, clinical evidence that intensive glycemic control reduces DKD is limited to the prevention of microalbuminuria and reduced progression to macroalbuminuria. Evidence of intensive glucose control effecting renal outcomes, including reduced eGFR or the doubling of plasma Cr levels, or on cardiovascular disease, is still ambiguous. Additionally, no reports have prospectively examined the effect of intensive blood glucose control on overt nephropathy with macroalbuminuria, and ESRD or CKD stage 4.

Risk of hypoglycemia

Recent clinical trials, including ADVANCE[46], ACCORD[48], and VADT[47], which reported HbA1c levels of 6.5%, 6.4%, and 6.9%, respectively, showed 1.5-3-fold increases in hypoglycemia in patients with type 2 diabetes who received intensive therapy to reach target glucose levels (with targeted HbA1c levels of < 6.5%, < 6.0%, and < 6.0%, respectively). However, intensive therapy did not decrease the risk of cardiovascular events. Moreover, in the ACCORD study[48], the mortality rates for patients treated with intensive therapy were significantly higher compared to conventional therapy patients. Although the source of the relationship between hypoglycemia and increased mortality in this study was unclear[49], hypoglycemia should be avoided. Therefore, glycemic control without hypoglycemia is important, and the use of glycemic control to target HbA1c levels should be considered in light of the risk factors pertinent to the individual patient, such as the presence of diabetic vascular complications, history of diabetes, and age. At the advanced stage of overt nephropathy with a reduction in renal functioning, the risk of hypoglycemia may be increased because of decreased gluconeogenesis in the kidney, changes in pharmacokinetics resulting from reduced renal function, and reduced insulin metabolism in the kidney. Therefore, it is necessary to select anti-diabetic medicines while considering the individual patient's renal functioning.

BLOOD PRESSURE CONTROL

Targeting blood pressure

Systolic blood pressure control is universally recommended in patients with diabetes to reduce the incidence of stroke, heart failure, diabetes-related death, and retinal photocoagulation, as well as to reduce the risk of the onset of microalbuminuria or progression to overt proteinuria. The early findings from the UKPDS suggest that a 10 mmHg decrease in systolic blood pressure is associated with a reduction of diabetic microvascular complications, including nephropathy, by 13%[50]. Additionally, in the ADVANCE study, a reduction of blood pressure from 140/73 mmHg (control group) to 136/73 mmHg (indapamide-perindopril group) was shown to reduce the risk of a major macro- or microvascular (mostly new microalbuminuria) event and mortality from any cause, including cardiovascular disease[51]. Therefore, the goal of blood pressure < 130/80 mmHg appears to be appropriate in type 2 diabetes to fight against the development and progression of DKD[52]. However, there are recent clinical guidelines for the management of high blood pressure in patients with diabetes and CKD. The KDIGO 2012 Clinical Practice Guidelines for the Evaluation and Management of Chronic Kidney Disease recommends targets for blood pressure in diabetes and CKD as follows. Blood pressure in diabetic adults with CKD and urine albumin excretion < 30 mg/24 h (or ACR < 30 mg/g Cr) should be treated to ≤ 140/90 mmHg, and blood pressure in diabetic adults with CKD and urine albumin excretion ≥ 30 mg/24 h (or ACR ≥ 30 mg/g Cr) should be treated to ≤ 130/80 mmHg. Moreover, the Standards of Medical Care in Diabetes 2014 of the ADA[33] recommends that people with diabetes and hypertension should be treated to < 140/80 mmHg, and lower systolic targets, such as < 130 mmHg, may be appropriate for certain individuals, such as younger patients. However, the 2014 Evidence-Based Guidelines for the Management of High Blood Pressure in Adults from the Panel Members Appointed to the Eighth Joint National Committee (JNC8)[53] recommend a blood pressure goal of < 140/90 mmHg in the population aged ≥ 18 years with CKD or/and diabetes. Thus, recommendations for blood pressure targets differ between the guidelines (Table 2); however, blood control targets should be considered with the risk of the individual patient, such as the presence or absence of other diabetic vascular complications, history of CVD and age,

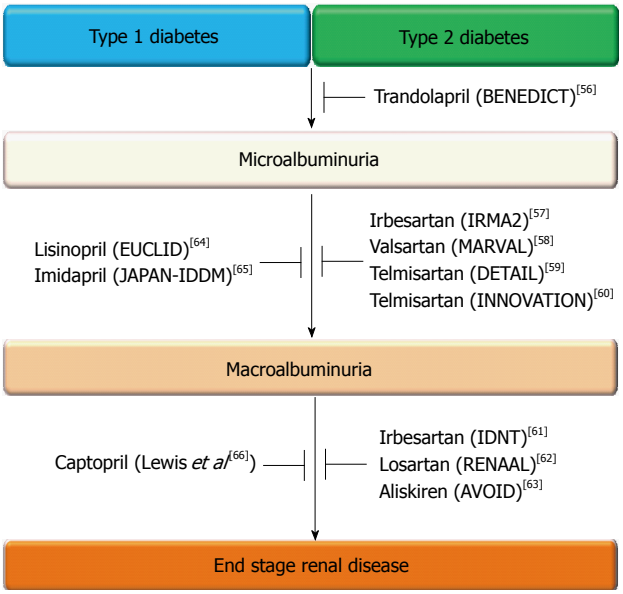

Figure 2 Beneficial effects of renin-angiotensin system inhibitors. Numerous landmark studies have shown the effectiveness of renin-angiotensin system inhibitors on diabetic kidney disease.

as well as glucose control targets.

ACE Inhibitors and ARBs

RAS activation is implicated in the pathogenesis of DKD. In diabetic patients with microalbuminuria or overt proteinuria, RAS inhibitors play a pivotal role in the prevention and treatment of DKD[54,55]. Landmark studies including type 1 and type 2 diabetic patients at various stages of DKD have provided abundant clinical evidence that treatment with RAS inhibitors, including ACE inhibitors and ARBs, slow the progressive decline of GFR, reduce micro- and macroalbuminuria, and reduce cardiovascular mortality and morbidity[54], as shown in Figure 2. Therefore, the use of RAS inhibitors for hypertension and albuminuria in diabetic patients is recommended as a first-line treatment[56-66].

Dual RAS blockade with an ACE inhibitor and ARB may be more effective in reducing proteinuria than monotherapy in patients with DKD. Based on the Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial, combination therapy with ramipril and telmisartan reduces proteinuria better than monotherapy; however, it worsens major renal outcomes, including dialysis, the doubling of serum Cr levels, and death[67,68]. Additionally, the Veterans Affairs Nephropathy in Diabetes Clinical Trials showed that combination therapy with an ARB (losartan) and an ACE inhibitor (lisinopril) in type 2 diabetic patients with macroalbuminuria significantly increased the risk of hyperkalemia and acute kidney injury[69]. Thus, combined RAS blockade should not be used in diabetic patients, especially elderly type 2 diabetic patients with normo- or microalbuminuria. First, an ACE inhibitor or ARB should be used, and its dosage should be increased to obtain an optimal anti-albuminuric or proteinuric response. Combination treatment with both an ACE inhibitor and an ARB should be prescribed by a nephrologist and given to patients with overt proteinuria or severe proteinuria, notwithstanding the use of the maximum dosage of the ACE inhibitor or ARBs. In such diabetic patients, monitoring of renal function is necessary, and treatment should be halted in the event of acute kidney injury, low blood pressure, or high potassium levels.

Mineralocorticoid receptor antagonists

Some clinical trials have demonstrated that treatment with spironolactone and eplerenone in addition to an ACE inhibitor or an ARB reduces proteinuria in patients with diabetes[70-75]. However, the long-term effect of mineralocorticoid receptor antagonists on GFR is not clear, and serum potassium levels should be monitored carefully.

Aliskiren

Aliskiren, a direct renin inhibitor, has been promoted for the suppression of DKD and cardiovascular disease. In the Evaluation of Proteinuria in Diabetes study[62], patients with DKD with overt proteinuria were treated with 100 mg of losartan, followed by the addition of a placebo or aliskiren (300 mg). Treatment with 300 mg of aliskiren reduced the mean urinary ACR compared with placebo treatment. However, the Aliskiren Trial in Type 2 Diabetes Using Cardio-Renal Endpoints study[76], which was performed to confirm the effectiveness of combination treatment with either an ACE inhibitor or an ARB plus aliskiren on both renal and cardiovascular events, was terminated because of adverse outcomes, including hyperkalemia and hypotension, and predicted futility in meeting the cardiovascular and renal endpoints.

Calcium channel blockers and diuretics

Because many hypertensive patients with DKD will require a combination therapy to adequately control blood pressure, commonly used combination therapies include an ACE inhibitor or an ARB plus a diuretic or a calcium channel blocker (CCB).

The Gauging Albuminuria Reduction With Lotrel in Diabetic Patients With Hypertension study tested the effect on albuminuria of initial combination therapy of either a dihydropyridine calcium channel blocker or a thiazide diuretic combined with the same ACE inhibitor in patients with type 2 diabetes and hypertension. In the study, both amlodipine and hydrochlorothiazide (HCTZ) combined with an initial treatment using benazepril decreased the median percent change in ACR from baseline to the end of the study; however, the benazepril plus HCTZ group had a greater reduction in albuminuria compared to the benazepril plus amlodipine group (median percent change in ACR: -72.1 *vs* 40.5, $P < 0.0001$)[77]. In contrast, the mean decrease in the eGFR during the observational period was less in the benazepril plus amlodipine group than in the benazepril plus HCTZ group (-2.03 ± 14.2 mL/min *vs* -13.64 ± 16.1 mL/min, $P < 0.0001$)[77].

The Avoiding Cardiovascular Events through Combination Therapy in Patients Living with Systolic Hypertension (ACCOMPLISH) trial was a randomized and double-blind trial in which 11506 patients with hypertension (60% of whom were diabetics) who were at high risk for cardiovascular events were assigned to receive treatment with either benazepril plus amlodipine or benazepril plus HCTZ. The benazepril-amlodipine combination had a relative risk reduction of 19.6% in cardiovascular events[78]. According to the sub-analysis of the ACCOMPLISH trial on renal outcomes, the events of CKD progression defined as a doubling of serum Cr concentration or ESRD (eGFR < 15 mL/min per 1.73 m^2 or need for dialysis) occurred at a frequency of 2.0% in the benazepril plus amlodipine group compared to 3.7% in the benazepril plus HCTZ group (HR = 0.52, 0.41-0.65, $P < 0.0001$). However, in the patients with CKD (more than half of patients have DKD), both the progression of CKD and cardiovascular mortality did not differ between groups[79].

It is still unclear which additional anti-hypertensive drug (CCB or diuretic) is better for providing both reno- and cardioprotection in DKD. Therefore, the risk of the individual patient, such as the history of CVD and age, should be taken into consideration.

LIPID CONTROL

Dyslipidemia, statins, and fibrates

Dyslipidemia is a major risk factor for atherosclerotic cardiovascular disease, which is a cause of mortality and morbidity in patients with diabetes and CKD[80,81]. In particular, low-density lipoprotein cholesterol (LDL-C) plays an important role in the development of coronary artery disease. Several clinical trials using statin-based lipid-lowering therapies in patients with CKD and diabetes have shown reductions in the risk of major atherosclerotic events. In addition to reducing the risk of cardiovascular diseases in CKD patients, evidence suggests that statin therapy in patients with predialysis CKD may slow the progressive loss of kidney function, measured as changes in urinary albumin/protein excretion or eGFR[82-89]. In the Collaborative Atorvastatin in Diabetes Study, atorvastatin (10 mg/d) treatment was associated with increased GFR in comparison with a placebo, and a modest beneficial effect was observed, particularly in patients with albuminuria. Moreover, atorvastatin was effective at decreasing cardiovascular disease (by 42%) in patients with a moderately decreased eGFR (30-60 mL/min per 1.73 m^2), and this treatment effect was similar to the 37% reduction in cardiovascular disease observed in patients without decreased eGFR[90]. Furthermore, a meta-analysis showed that statin therapy was associated with decreased albuminuria compared to a placebo[87].

The Fenofibrate Intervention and Event Lowering in Diabetes study demonstrated that fenofibrate (200 mg/d) reduced cardiovascular events, reduced albuminuria, and slowed eGFR loss over 5 years, although it initially and reversibly increased plasma Cr levels. In a meta-analysis, fibrates reduced the risk of albuminuria progression in patients with diabetes and reduced the risk of major cardiovascular events and cardiovascular death in patients with an eGFR of 30-59.9 mL/min per 1.73 m^2[91,92].

Statins and fibrates can exert renoprotective effects pleiotropically, such as anti-oxidant, anti-inflammation, and anti-fibrotic effects, independent of their lipid-lowering effects, in experimental animal models[93,94].

KDOQI guidelines and the ADA recommend that the LDL-C target in patients with diabetes or/and CKD should be < 100 mg/dL, and a lower LDL-C goal of < 70 mg/dL is a therapeutic option in individuals with overt CVD, by treatment with statins. Triglyceride levels < 150 mg/dL and high-density lipoprotein cholesterol (HDL-C) > 40 mg/dL in males and > 50 mg/dL in females are desirable[33,35].

MULTIFACTORIAL INTENSIVE THERAPY

Effects on the progression of diabetic kidney disease

The Steno-2 study showed the effect of multifactorial intensive therapy on the progression of nephropathy in patients with type 2 diabetes[95]. In this study, 160 patients with type 2 diabetes and microalbuminuria (average age, 55 years) were randomly divided, with 80 patients assigned to a standard therapy group and 80 patients assigned to an intensive therapy group. The progression of nephropathy was evaluated as a secondary end point. During the 1993-1999 period, the targets for glycemic control, systolic blood pressure, diastolic blood pressure, total cholesterol levels, and triglyceride levels were < 6.5%, < 140 mmHg, < 85 mmHg, < 190 mg/dL, and < 150 mg/dL, respectively, in the intensive therapy group. Patients were administered ARB or ACE inhibitors (regardless of their blood pressure); patients with ischemic heart disease or peripheral vascular disease were given aspirin, and supplementation with vitamin C and E was also provided. Additionally, diet therapy (lipid restriction, < 30% of energy intake per day and < 10% from saturated fatty acid intake) and exercise therapy (3-5 times/wk, moderately intense activity) were prescribed. In the 2000-2001 period, the targets for fasting total cholesterol levels, systolic blood pressure, and diastolic blood pressure were changed to < 175 mg/dL, < 130 mmHg, and < 80 mmHg, respectively, because the treatment guidelines in Denmark changed. In the average observation period of 7.8 years, HbA1c; systolic and diastolic blood pressure; total cholesterol, LDL-C, and triglyceride levels; and fat intake were significantly reduced in the intensive therapy group compared with the standard therapy group. Moreover, the use of aspirin was significantly higher in the intensive therapy group, and urinary albumin excretion was significantly decreased in the intensive therapy group (46 mg/d) compared with the standard therapy group (126 mg/d). Moreover, the risk of onset and progression of nephropathy was reduced to a hazard ratio of 0.39 (CI: 0.17-0.87).

Furthermore, after the Steno-2 study, 63 patients in the standard therapy group underwent intensive therapy with 67 patients of the intensive therapy group in the average follow-up period of 5.5 years[96]. In the follow-up study, the onset and progression of nephropathy were assessed as secondary endpoints. At the end of the follow-up period, glucose, blood pressure, and lipid control in the standard therapy group were improved to almost the same levels as in the intensive therapy group. However, for the total observation period of 13.3 years combined with an average follow-up period of 7.8 years, the onset and progression of nephropathy were decreased in the intensive therapy group [HR = 0.44 (CI: 0.25-0.77)]. Six cases and one case progressed to ESRD in the standard and intensive therapy groups, respectively ($P = 0.04$).

Additionally, a cohort study with a 4-year follow-up of 1290 type 2 diabetic patients with normal albuminuria was performed using multifactorial intensive therapy[97]. In this cohort study, the targets of blood glucose, blood pressure, LDL and triglyceride levels were as follows: HbA1c < 7.0%, < 130/80 mmHg, < 100 mg/dL, < 150 mg/dL, and HDL ⩾ 40 mg/dL (male) per 50 mg/mg per deciliter (female). New microalbuminuria appeared in 211 patients (16.4%) and HbA1c levels < 7% (HR = 0.729, 95%CI: 0.553-0.906, $P = 0.03$), blood pressure < 130 mmHg [HR = 0.645 (CI: 0.491-0.848), HDL ⩾ 40 mg/dL (male) per 50 mg/dL (female), HR = 0.715 (CI: 0.537-0.951)] were associated with the onset of albuminuria.

Accordingly, multifactorial intensive therapy is recommended for suppressing the onset and progression of early diabetic nephropathy; however, it should be noted that this recommendation is based on a small RCT. Moreover, the suppressive effect of multifactorial intensive therapy on nephropathy is not clear in the advanced stage of overt nephropathy.

Effects on the onset of cardiovascular events

In the Steno-2 study described above, the incidence of cardiovascular diseases, including cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, revascularization, and amputation, were evaluated as the primary endpoints over 7.8 years[95]. Thirty-three cardiovascular events (24%) in 19 cases were observed for the intensive therapy group; conversely, 35 cardiovascular events (40%) were observed in the standard therapy group. These results indicate that the risk of cardiovascular disease in type 2 diabetic patients with microalbuminuria was significantly reduced after multifactorial intensive therapy compared with standard therapy [HR = 0.47 (CI: 0.24-0.73)].

In the Steno-2 follow-up study, performed for an average of 5.5 years in addition to the original 7.8 years, the incidence of lower limb amputation, nonfatal stroke, nonfatal myocardial infarction, coronary artery bypass grafting, and percutaneous transluminal coronary angioplasty were assessed as the primary endpoints[96]. At the end of the follow-up period, glycemia, blood pressure, and lipid control for the standard therapy group had improved to levels similar to those found in the intensive therapy group. However, for the total observation period of 13.3 years, the onset of cardiovascular disease was decreased in the intensive therapy group. In addition, there were 48 cases and 158 cardiovascular events in the standard therapy group, in contrast to 28 cases and 51 cardiovascular events in the intensive therapy group.

Remission and regression of albuminuria

Reduction of microalbuminuria in diabetic patients occurred more frequently than we expected. Araki *et al*[98] reported that microalbuminuria in type 2 diabetic patients could improve to normoalbuminuria (remission) or could decrease by more than 50% from the baseline (regression) based on the results of a prospective observational follow-up study over a 6-year period. The 6-year cumulative incidence of progression from microalbuminuria to overt proteinuria was 28% (95%CI: 19%-37%), whereas the remission and regression rates were 51% (95%CI: 42%-60%) and 54% (95%CI: 45%-63%), respectively (Figure 2). In a pooled logistic regression analysis, each modifiable factor was trisected according to the number of patients and was applied as three categories in the analysis. The results showed that microalbuminuria of short duration, the use of RAS blockade, HbA1c < 7.35%, and lower systolic blood pressure (< 130 mmHg) were identified as independent factors associated with remission/regression of microalbuminuria.

ARBs have also been shown to induce remission and regression of microalbuminuria in type 2 diabetic patients. In the Incipient to Overt: Angiotensin Ⅱ Blocker, Telmisartan, Investigation on Type 2 Diabetic Nephropathy study, remission of microalbuminuria at the final observation point occurred in 21.2% of patients treated with 80 mg of telmisartan, 12.8% of patients treated with 40 mg of telmisartan, and 1.2% of patients given a placebo (both telmisartan doses *vs* placebo, $P < 0.001$)[58]. Additionally, patients receiving 80 or 40 mg of telmisartan achieved superior renoprotection, as indicated by lower transition rates to overt nephropathy compared to the placebo patients. Taken together, these results strongly indicate that RAS blockade using an ARB not only prevents the progression of microalbuminuria to overt proteinuria but also induces remission and regression of microalbuminuria in type 2 diabetic patients.

The Steno-2 study also demonstrated that a high proportion of patients with microalbuminuria returned to normoalbuminuria through the multifactorial intervention. After a mean of 7.8 years of follow-up, 46 (31%) patients returned to normoalbuminuria, 58 (38%) patients still had microalbuminuria, and 47 (31%) patients progressed to overt proteinuria[99]. Lower HbA1c levels, initiation of antihypertensive therapy, and initiation of RAS inhibitors during the follow-up period were independently associated with remission of microalbuminuria. A recent analysis focusing particularly on the effect of lowering blood pressure clearly showed that more than half of all type 2 diabetic patients with microalbuminuria and macroalbuminuria returned to normoalbuminuria with receiving any blood pressure-lowering drugs in the

ADVANCE study[100]. However, more patients achieved remission to normoalbuminuria in the perindopril-indapamide treatment group than in the placebo treatment group.

Clinical impact of the remission and regression of albuminuria on cardiovascular events and kidney function

The clinical impact of the remission and regression of microalbuminuria was demonstrated by the observed reduction in the risk of renal and cardiovascular events during an expanded 2-year follow-up (beyond the initial 6 years of the study reported by Araki *et al*[101], described above). The primary outcome measure consisted of "combined incidence," defined as cardiovascular death by and first hospitalization for renal and cardiovascular events. A secondary outcome was kidney function, as determined by the annual decline of eGFR. During the total 8-year follow-up period, 47 patients experienced primary renal and cardiovascular events. Eleven first occurrences of outcomes occurred in subgroups that achieved remission of microalbuminuria; in contrast, 36 such events were observed for the non-remission group. The pooled logistic analysis, adjusted for sex, age, initial ACR levels, history of cardiovascular disease, current smoking, HbA1c level, total cholesterol level, blood pressure, use of RAS inhibitors, use of lipid-lowering drugs, and body mass index, showed that the relative risk for outcomes in patients who achieved remission was 0.25 (95%CI: 0.07-0.87) compared with those whose microalbuminuric status did not change during the follow-up period, whereas the relative risk for patients who progressed to overt proteinuria was 2.55 (95%CI: 1.04-6.30) (Figure 2). First occurrences of these outcomes were classified into subgroups defined by achieving a reduction greater than 50% in urinary albumin excretion in the course of 12 events for the regression group and in 35 events in the non-regression group; these patients were labeled as having failed to achieve remission.

Kaplan-Meier estimations showed that the cumulative incidence of evaluated events was significantly lower in the regression group than in the non-regression group. The 8-year cumulative incidence of these outcomes in the regression group showed a 59% decrease compared to the non-regression group. The adjusted risk for outcomes in patients who achieved regression was 0.41 (95%CI: 0.15-0.96) compared with those whose microalbuminuric status did not show regression during the follow-up. As anticipated, the annual decline of eGFR for the progression group (median: 4.2 mL/min per year) was significantly faster than that for the non-change group (2.4 mL/min per year), whereas the annual decline of eGFR for the remission group was significantly slower (1.1 mL/min per year) and was almost identical to the decline experienced through normal aging reported in healthy people[102].

The effect of reducing microalbuminuria on kidney functioning was also shown in a secondary analysis of the Steno-2 study[101]. The patients who reverted to normoalbuminuria had an average eGFR decrease of 2.3 mL/min per year; however, those who still had microalbuminuria experienced an average eGFR decrease of 3.7 mL/min per year, and those who progressed to overt proteinuria showed the highest eGFR decline of 5.4 mL/min per year. These results show that remission of microalbuminuria is closely related to the improved renal functioning over the long term.

OTHER PROSPECTIVE THERAPEUTIC STRATEGIES

Vitamin D receptor activation

Stimulation of vitamin D receptors exerts protective activity through multiple mechanisms, including inhibition of the RAS, regulation of proliferation and differentiation, reduction of proteinuria, anti-inflammation, and anti-fibrosis[103]. Growing evidence indicates that vitamin D exerts anti-proteinuric and renoprotective effects in DKD patients. The VITAL study demonstrated that treatment with paricalcitol, a selective vitamin D receptor activator, reduced urinary albumin excretion in type 2 diabetic patients treated with RAS inhibitors[104]. Additionally, Kim *et al*[105] showed beneficial effects of vitamin D (cholecalciferol) repletion on urinary albumin and transforming growth factor-β_1 excretion in type 2 diabetic patients with CKD undergoing established RAS inhibition therapy; similar effects were also observed in the VITAL study. Treatment with cholecalciferol led to significantly higher levels of circulating 25(OH)D and $1,25(OH)_2D_3$ relative to baseline, and increased levels of active forms of vitamin D were correlated with a decrease in urinary ACR and TGF-β_1 at the end of a 4-mo intervention period. These data indicate that vitamin D compounds may be useful tools for delaying the progression of DKD beyond the effects expected from established RAS inhibition protocols.

Uric acid-lowering drugs

Multiple longitudinal cohort studies have shown that elevated serum uric acid levels are associated with a higher risk of the onset and progression of microalbuminuria in addition to sustained decline of GFR among type 1 diabetic patients[106-108]. In a cohort study of 263 newly diagnosed type 1 diabetic patients performed by the Steno Diabetes Center group[106], serum uric acid levels measured shortly after the onset of type 1 diabetes were a significant independent predictor of macroalbuminuria 18 years later (HR = 2.37, 95%CI: 1.04-5.37, P = 0.04). Additionally, the Coronary Artery Calcification in Type 1 Diabetes study showed that serum uric acid levels predicted the transition from microalbuminuria to macroalbuminuria[107]. In 324 type 1 diabetic patients, every 1 mg/dL increase in uric acid levels at baseline was associated with an 80% increase in the predicted odds ratio of developing microalbuminuria or macroalbuminuria after 6 years of follow-up (OR = 1.8, 95%CI: 1.2-2.8, P = 0.005). A 6-year follow-up of a prospective cohort study

of type 1 diabetic patients without proteinuria conducted by the Joslin Diabetes Center demonstrated a significant association ($P < 0.0002$) between serum uric acid and an early decrease in GFR, defined as a GFR cystatin decrease exceeding 3.3% per year[108]. When baseline uric acid concentrations were treated categorically (in mg/dL: < 3.0, 3.0-3.9, 4.0-4.9, 5.0-5.9, and ≥ 6), the risk of early decrease in GFR increased linearly (9%, 13%, 20%, 29%, and 36%, respectively). This linear increase corresponds to an OR of 1.4 (95%CI: 1.1-1.8) per 1 mg/dL increase in uric acid levels.

Furthermore, a post-hoc analysis of the Reduction of Endpoints in non-Insulin Dependent Diabetes Mellitus with the Angiotensin Ⅱ Antagonist Losartan trial showed that the decrease in serum uric acid levels induced by losartan accounted for 20% of the renoprotective benefit provided by this medication[109]. However, it is not clear whether reducing uric acid levels could prevent or delay GFR decline in diabetic patients who are at high risk for the progression of DKD; therefore, clinical trials are necessary to elucidate the beneficial effects of uric acid-lowering medicine on preventing DKD.

GLP-1 receptor agonists and DPP-4 inhibitors

Incretin-related therapies, including dipeptidyl peptidase (DPP)-4 inhibitors and glucagon-like peptide (GLP)-1 receptor agonists, have been developed as one of the most promising treatments for type 2 diabetes because of their effectiveness at reducing glucose levels with a low risk of hypoglycemia and no weight gain[110-112]. DPP-4 inhibitors increase the concentration of endogenous incretins, such as GLP-1 and glucose-dependent insulinotropic polypeptides, and GLP-1 analogues that are not degraded by DPP-4 may stimulate GLP-1 receptors in turn. Stimulation of GLP-1 receptors increases glucose-dependent insulin secretion from pancreatic β-cells and suppresses glucagon release from α-cells, leading to improved glucose control[110]. In addition to its action on the pancreas, GLP-1 may have direct effects on other cells and tissues, including the kidney, heart, and blood vessels, *via* stimulation of the GLP-1 receptor[113,114], independent of its glucose-lowering effects.

The GLP-1 receptors in the kidney are expressed in the glomerular endothelial cells, mesangial cells, and proximal tubular cells[115-120], and previous reports have shown that the expression of GLP-1 receptors decreases in the diabetic kidneys of animal models[115]. The renoprotective effect of GLP-1 may be accomplished through anti-inflammation[116], anti-oxidants mediated through cyclic AMP-mediated protein kinase A activation[117,120], or blood pressure regulation *via* sodium handling in proximal tubular cells[121]. DPP-4 is expressed in renal tubular cells, especially in the brush-border and microvillus fractions, podocytes, and endothelial cells[122,123]; however, the physiological role of DPP-4 in the kidney has not been elucidated. Previous reports have shown that DPP-4 expression is increased in the diabetic kidneys of animal models[124]. DPP-4 is a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides. Therefore, DPP-4 cleaves not only incretins but also many substrates, such as cytokines, chemokines, hormones, and neuropeptides[125]. Among these substrates, high-mobility group protein-B1, meprin β, and neuropeptide Y have been identified as candidate targets for GLP-1-independent effects of DPP-4 inhibitors in the kidneys[114].

Several clinical studies have shown beneficial effects of DPP-4 inhibitors[126,127] and GLP-1 analogues[128] on albuminuria in type 2 diabetic patients. Recent reports have demonstrated that linagliptin administration in addition to stable RAS inhibition leads to a significant reduction in type 2 diabetes with albuminuria and renal dysfunction, independent of changes in glucose levels or systolic blood pressure[129]. Further studies, including randomized controlled clinical trials in large populations, are necessary to confirm the long-term effects of incretin-related medicines in DKD.

CONCLUSION

Reduced microalbuminuria may be frequent in diabetic patients. Physicians have to care for these diabetic patients with an aggressive multifactorial management plan as early as possible after the development of microalbuminuria. This multifactorial management regimen includes glycemic control without triggering hypoglycemia, blood pressure control using RAS inhibitors, and lipid control using statins or fibrates. In addition to these therapies, vitamin D receptor activators, uric acid-lowering drugs, and incretin-related drugs for glycemic control are promising therapies for stopping the progression of DKD. However, in the future, the development of novel therapies that not only function to prevent renal decline but also simultaneously attenuate CVD are necessary because the current multifactorial treatment is not still enough.

The remission or regression of microalbuminuria results in reduced risk of both renal and cardiovascular events; therefore, albuminuria is a useful biomarker for the diagnosis of DKD and the assessment of therapeutic effects for DKD. However, some patients with diabetes have advanced renal pathological changes and progressive kidney function decline even though urinary albumin levels are in the normal range, indicating that albuminuria is not the perfect biomarker for early detection of DKD[130]. Recent studies have provided some possible new markers for DKD in type 1[131,132] and type 2 diabetic patients[133]. Serum concentrations of the soluble receptors 1 and 2 for Tissue Necrosis Factor (sTNFR1 and sTNFR2) had a stronger correlation with decline in GFR than urinary ACR[131,132]. sTNFR1 was associated with the development of ESRD in type 2 patients during a 12 year follow-up[133]. However, additional clinical data about such new biomarkers for the early diagnosis and prediction of DKD should be accumulated, and at the same time, it is necessary to determine whether the new biomarker is a

predictive marker for CVD.

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P- Reviewers: Cui WP, Lehtonen SH, Schaalan M
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diabetic nephropathy and inflammation

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Received: December 5, 2013 Revised: February 11, 2014
Accepted: April 17, 2014
Published online: June 15, 2014

Abstract

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure worldwide. Besides, diabetic nephropathy is associated with cardiovascular disease, and increases mortality of diabetic patients. Several factors are involved in the pathophysiology of DN, including metabolic and hemodynamic alterations, oxidative stress, and activation of the renin-angiotensin system. In recent years, new pathways involved in the development and progression of diabetic kidney disease have been elucidated; accumulated data have emphasized the critical role of inflammation in the pathogenesis of diabetic nephropathy. Expression of cell adhesion molecules, growth factors, chemokines and pro-inflammatory cytokines are increased in the renal tissues of diabetic patients, and serum and urinary levels of cytokines and cell adhesion molecules, correlated with albuminuria. In this paper we review the role of inflammation in the development of diabetic nephropathy, discussing some of the major inflammatory cytokines involved in the pathogenesis of diabetic nephropathy, including the role of adipokines, and take part in other mediators of inflammation, as adhesion molecules.

Key words: Diabetic Nephropathy; Inflammation; Albuminuria; Adhesion molecules; Cytokines

Core tip: In recent years, new pathways involved in the development and progression of diabetic kidney disease have been elucidated; accumulated data have emphasized the critical role of inflammation in its pathogenesis. Expression of cell adhesion molecules, growth factors, chemokines and pro-inflammatory cytokines increased in renal tissues of diabetic patients, and serum and urinary levels of cytokines and cell adhesion molecules, correlated with albuminuria. We review the role of inflammation in the development of diabetic nephropathy, discussing some of the major inflammatory cytokines involved in its pathogenesis, including the role of adipokines, and other mediators of inflammation, as adhesion molecules.

Original sources: Duran-Salgado MB, Rubio-Guerra AF. Diabetic nephropathy and inflammation. *World J Diabetes* 2014; 5(3): 393-398 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/393.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.393

INTRODUCTION

Diabetes mellitus (DM) is the leading cause of chronic renal failure in development countries and is increasing as a cause of morbility and mortality worldwide. Both type 1 and 2 diabetes, but principally the last one, plays an important role in this problem because of the impact of its complications[1-4].

Among all these complications, diabetic nephropathy (DN) has become the principal cause of end-stage renal failure and cardiovascular mortality, this condition appears after many years of diabetes beginning[3,5].

It is well understood that type-2 DM is not an immune disease but at this time we could consider that there is evidence that the combine of immunologic and inflammatory mechanisms play a pivotal role in its presentation, development and finally its progression.

The DN take place nearby one-third of patient with type 1 DM and 25% approximately of patients with type 2[4,6].

In México, it is described that the main cause of chronic renal failure is type 2 DM, nevertheless we know that not all diabetic patients develop DN, moreover glucose control is not a warranty of a life free of microangiopathic complications[7].

It has been found that despite all pharmacologic therapies available for DN treatment, some patients develop kidney damage, that is why the need of complete understanding of molecular, metabolic and environmental factors that lead to DN and their interaction between them.

Among diverse factors that could interact actively in pathogenesis and progression of DN have been studied the age, gender, smoking, hypertension and hyperuricemia, all of them with suggestive results of correlation with renal disease[2].

In this paper we review the inflammatory factors that lead to the development and progression of DN.

PHYSIOPATHOLOGY

DN is characterized by glomerular hypertrophy, thickness of basement, tubular and glomerular membranes and accumulation of extracellular matrix in these membranes that finally cause tubulointerstitial and glomerular fibrosis and sclerosis[2,6,8]. As we can see several kidney structures are susceptible to hyperglycemia, and this metabolic change cause organ damage due to several cellular *via* including genetic activation and expression, advanced glycation end products generation, polyol pathway activation, abnormal protein kinase activation (PKC), raise of oxidative stress and the molecules that act as growth factors, transcription factors and others[4,8].

There is a response for hyperglycemia from the system, the transcription factors regulate the gene encoding some cytokines like transforming growth factor β (TGF-β), chemokine C-C motif ligand 2, fibronectin, osteopontin, decorin, thrombospondin, aldose reductase and plasminogen activator inhibitor 1, all these molecules involved in inflammation, extracellular matrix synthesis and its degradation are increased in type-2 DM[4].

Some other factors in relation to DN, it is known that some metabolic *via* activated by hyperglycemia are not enough to cause the kidney complication. The family predisposition to disease, race and other environmental factors interact with hemodynamic changes producing, as a result, advanced glycation end products, glucose reduction and sorbitol accumulation into the cell, overproduction of reactive oxygen species and activation of signaling *via* as PKC and mitogen-activated protein kinase[2].

Diabetic patients then could have albuminuria since early phases or stages of organ damage, it is also considered as a very sensible marker of kidney disease progression. As a result there are many glomerular abnormalities including podocyte structure alteration, reduction of nephrin expression and increase of filtration rate, a hallmark of DN[9].

Many mechanisms were investigated in this process, for a better understanding these are divided in mechanisms of immune cell infiltration of kidney, molecules involved in progression and intracellular pathways activated in DN.

Role of inflammation

Now we know that activation of the immune system and chronic inflammation are both involved in pathogenesis of DM and as a result DN. Some studies have demonstrated that cytokines, chemokines, growth factors, adhesion molecules, nuclear factors as well as immune cells as monocytes, lymphocytes and macrophages are all involved in DM pathogenesis and of course play an important role in DM complications[1,5].

IMMUNE CELLS

Macrophages

Macrophages are recognized as the principal inflammatory cell involved in kidney damage, their accumulation relates with severity of DN in experimental models[3].

These cells are responsible of the calling "renal remodeling", so therapeutics proposed to inhibit their accumulation may help to stop progression.

Two subtypes are mainly involved in DN, M1 macrophages activated by Th1 cells, that are able to increase inflammatory response by cytokines expression [interleukins, tumor necrosis factor (TNF) and interferon γ]; and M2 macrophages activated by Th2 cells that promote tissue repairmen, remodeling and neovascularization by antiinflammatory cytokines expression[3]. Is in this way that investigations are working, it is known that the macrophage subtype levels related with recruitment of circulating monocytes from vascular space to glomerular tissue.

Meanwhile M1 macrophages enhance inflammatory response by upper production of reactive oxygen species (ROS), this point will be reviewed later.

As to activated M2 macrophages, they help in inflammation ending with the participation of interleukin 10 (IL-10), TGF-β1, both with anti-inflammatory functions. Besides they produce proinflammatory factors as chemokines, cytokines and superoxide anions[3].

Many investigations are directed to show that statins are capable to block M1 macrophage actions but at the same time improve M2 functions. It will be helpful as one of the strategies used in the treatment of DN directed to this point.

T lymphocytes

T lymphocytes play a determinant role in early kidney damage in DN, they have cytotoxic effects besides macrophages tissue activation[3].

The first contribution of the studies was about the increase in local accumulating T cells in diabetic experimental models. Xiao *et al*[10] and Moon *et al*[11] showed an increase in CD4 and CD8 lymphocytes in diabetic mouse, these changes were observed in glomeruli and interstice.

In type 1 DM there is an increase of T lymphocytes

in juxtaglomerular tissue that results in a disturbance in albumin glomerular excretion and a decrease of renal filtration. Many other studies have shown at this time that T lymphocytes systemic, specifically circulating CD8, correlated with albuminuria[6].

Lei *et al*[6] demonstrated with a multiple regression analysis a positive association between lymphocytes CD8 and albuminuria in type 2 DM patients and the cell activation could be a systemic response.

Several metabolic and genetic *via*, may activate systemic T lymphocytes. In type 2 DM those cells may be activated by hemodynamic, environmental and metabolic changes. The most important activation seen due to hyperglycemia, that activates nuclear factor κB and this results in an over stimulation of lymphocytes by specific cytokines as IL-12 produced by macrophages, and then, production of interferon further lymphocyte activation[6].

CHEMOKINES

These molecules are active components of inflammatory cells recruitment in kidney and are present in every phase of kidney damage[8].

Many chemokines are involved in the inflammatory response in DN, monocyte chemoattractant protein (MCP-1) was first described in its role in early phases of atherosclerosis[12].

MCP-1

MCP-1 can promote transformation of monocytes in macrophages, the last ones produce diverse cytokines as IL-6 and TNF-α, both induce atherosclerosis changes in vascular walls that results in illness progression. Because of its expression is as high in the atherosclerotic plaques than in impaired plaques, systemic MCP-1 was measured in many studies in order to show an association between this chemokine and DN markers. Takebayashi *et al*[12] found that patients with urinary albumin excretion presented higher circulating levels of MCP-1 than patients without this alteration.

All these findings could suggest that MCP-1 plays an important role in pathogenesis of DN as the protein produced not only in vascular wall, atherosclerotic plaques but also in tubular epithelial cells.

CYTOKINES

Cytokines are molecules with a wide spectrum of physiological actions, many of them due to their pleiotropic actions. They have capacity to combine actions in order to amplify their effects and then induce synthesis or expression of other cytokines if needed.

In 1991 it was suggested for the first time the participation of cytokines with inflammatory actions in the development of DN, by demonstration of high production of these molecules from macrophages in glomerular membranes from diabetic rats, but not from non-diabetic rats[5].

At this time we now that inflammatory cytokines play an important role in DN, but cytokines have been involved in the development of other microangiopathic complications of DM[1].

Interleukins

Interleukins are a group of cytokines produced by many cells in different tissues. According to their physiologic actions, they are classified as antiinflammatory and proinflammatory molecules[3].

IL-1

Many studies have shown that IL-1 promotes an increase of adhesion molecules in glomerular endothelium as well as expression of these molecules in other kidney structures[1].

Mesangial cells and renal tubular epithelium overexpress intercellular adhesion molecule-1 (ICAM-1) and E-selectin, additionally, IL-1 induces prostaglandin E2 synthesis in mesangial cells, this fact cause alterations in the glomerular hemodynamics[1].

Moreover, IL-1 stimulates hyaluronan synthesis, leading to cell proliferation in DM patients, this facts contributes to development of DN. It is known that this proinflammatory cytokin is increased in experimental models with albuminuria and at the same time with macrophages accumulation[1]. According to these pathological changes, IL-1 modifies vascular permeability and increase expression of chemokines that as a result leads proliferation and synthesis of extracellular matrix in mesangium[3].

IL-6

IL-6 is another molecule that has been studied in DN due to its pleiotropic effects. Many authors showed that IL-6 concentration is increased in DN. IL-6 has a direct effect in glomerular and infiltrating cells, this effect modified extracellular matrix dynamics affecting membrane thickening in renal glomeruli[1,3].

IL-6 is a cytokine that can enhance proliferation, overexpression of extracellular matrix and affect vascular permeability; these actions lead to DN progress[1].

It has been shown that serum IL-6 is increased in patients with type 2 DM with nephropathy[3].

IL-18

The principal actions of this inflammatory cytokine are; to enhance the production of other inflammatory cytokines by mesangial cells, and upregulation of ICAM-1. Its serum concentration is increased in DN as well as other interleukins and has a determinant role in endothelium apoptosis[1].

IL-18 has several sources in the diabetic kidney as infiltrating, T-lymphocytes, macrophages, monocytes as well as proximal tubule cells. There is a direct correlation between IL-18, albuminuria and albumin excretion rate, so it's relationship with nephropathy has been identified[13].

TNF-α

This is an inflammatory cytokine with many determinant

actions in inflammatory response by several tissues and pleiotropic effects. TNF-α is produced by infiltrating cells, as monocytes, macrophages and T lymphocytes, as well as kidney cells. Previous reports shown that TNF-α can be stored as a proactive form[1].

Its actions are widely known as systemic and in many cases direct cytotoxic effect in kidney cells principally. Nevertheless actions as activation of second messengers, transcription factors (TF), growth factors, cell adhesion molecules, express or synthesis of cytokines and others are recognized as variable biological effects of this molecule, of course all of them playing a determinant role in DN pathogenesis[1].

When TNF-α binds to the receptors, several signaling pathways are activated and a cascade of molecules begin their expression in renal cells, many of this actions results in apoptosis and necrosis[5].

The negative effects have been described in experimental models and in humans[1]. Those effects were manifested as DM nephropathy, hypertension, nephritis and glomerulonephritis, this fact could be demonstrated with the correlation found by Navarro-González *et al*[5]. in 2005 between renal TNF-α and albumin excretion in diabetic mice. This observation demonstrated that this inflammatory molecule is directly involved in pathogenesis of DN by leading cell and tissue damage; moreover albuminuria has been related to a enhanced stimuli for overexpression of TNF-α[3].

TNF-α alters glomerular hemodynamics and promotes increased vascular endothelium permeability. Infiltration by inflammatory cells, neo-formation of extracellular matrix, production of ROS and blood flow disturb are others recognized effects of TNF-α in renal structures[1].

TGF-β1

TGF-β1 is a cytokine member of TGF-β1 superfamily considered also as a transcription factor related to development of renal damage by promoting renal fibrosis. Its activity is recognized as inflammatory and fibrogenic, with two isoforms, TGF-β2 and TGF-β3, all produced by kidney cells, the union between this cytokine and its receptor phosphorylate the Smads. Smads are intracellular proteins that transduce extracellular signals from TGF-β ligands to the cellular nucleus and activate downstream gene transcription. This family is considered to be involved in development of inflammation and fibrosis in the kidney[4,8,13].

That is why TGF-β1 is recognized as one of the principal mediators of structural changes seen in DN, its concentration is higher in DM patients with urinary albumin excretion than in normal individuals[8].

The upregulation of TGF-β1 promotes extracellular matrix proliferation and at the same time inhibits the degradation, so that is why actually overexpression of this factor is directly associated with severe forms of glomeruloesclerosis and glomerulonephritis[8]. Some other changes are favored by TGF-β1, for example the induction of transforming epithelial cells of tubules into fibroblasts; this process is responsible of renal fibrosis, a result of persistent inflammation.

TGF-β1 is considered too as a cytokine which principal function in inflammation is to inhibit this process. Letterio *et al*[14] discovered that experimental models with impairment in *TGF-β1* gene are highly susceptible to several inflammation resulting in autoimmune diseases and even death[15,16].

El Mesallamy *et al*[8] correlated TGF-β1 concentrations with Connective tissue growth factor level; their findings showed that between these two molecules there is a closed interaction in DN. So as we can see, TGF-β1 is a molecule that can regulate not only its own release and its actions but also it has the ability to modulate other molecular releases and their interactions in signaling pathways.

It seems like TGF-β1 has a complex role in renal inflammation, we know that this protein is present as active and as a latent forms, the first one is related to mediator of renal fibrosis that can progress according to many other factors. The second form is a protective factor for the development of renal damage. Some mechanisms for these findings are not well understood yet[17].

TF

Proteins known as TF bind themselves to some gene specific regions to activate or inhibit nuclear transcription process[4].

TF were classified according to its main action, they can be constitutively active or regulatory factors and they can be activated by several metabolic and environmental stimuli in many cellular sites. Due to this last point we can subclassify TF in nuclear factors, cytoplasmic factors and steroid receptor superfamily[4].

Several TF are involved in DN development, here we have the most relevant.

Upstream stimulatory factors 1 (USF1) and USF2 are a part of Myc family and encoded by two different genes.

USF1 and USF2 are involved in some glucose genes responses in many types of cells including kidney cells. It has been shown that overexpression or increase in concentration of these TF are related with albuminuria development and even more the upregulation of many other molecules with proved actions in DN pathogenesis[4].

Smads

Smads conform a transcription factor family that regulates the expression of certain genes. Three classes are known: the receptor-regulated Smads (R-SMAD) which include SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8/9; the common-mediator Smad (co-SMAD) which includes only SMAD4, which interacts with R-SMADs to take part in signaling and the antagonistic or inhibitory Smads which include SMAD6 and SMAD7, they block the activation of R-SMADs and co-SMADs[17].

As mentioned before this family is closely involved with TGF-β1, which phosphorylate Smad 2 and Smad 3 to form a complex with Smad 4, all this process leads to regulate gene in cell nuclei[17].

Smad 4 is the most related with inflammation, if there is an abnormality of this protein, the inflammatory response is more intense and leads a higher concentration of diverse cytokines and adhesion molecules.

There is another relationship that leads the process to be functional for kidney, this happens when TGF-β1 regulates Smad 7 transcription by Smad 3 and Smad 4 binding, so, when Smad 4 is impaired we can see and exaggerated inflammatory response for reduction of Smad 7 expression, activation of Nuclear Factor κB and fibrosis inhibition[17].

Smad 4 seems to be a key point in regulation of TGF-β1 and its different functions media the conjunction with Smad 7 and Smad 3 expression in kidney.

The case of Smad 7 is quiet interesting, it acts in an inhibitory way and regulates the active function of Smad 2 and Smad 3 but by a negative feedback.

The Smad 7 expression is enhanced by TGF-β1 that in normal condition has a negative feedback inhibit the action of Smad and at the same time degrade this transcription factors. When Smad 7 gets degraded then kidney fibrosis begins. If Smad 7 decline renal inflammation persists and as a result begins fibrosis *via* TGF-β and Smad 3.

In as much as the pivotal role of Smad 7 some investigators decided to study therapeutic effects of this factor in experimental models. When Smad 7 was transferred to kidney they found that if there is an overexpression of Smad 7, inflammation and fibrosis decrease.

Adhesion molecules

ICAM-1 and vascular adhesion molecule-1 (VCAM-1) are involved in the attachment of leukocytes to the vascular wall and penetration into the intima, once there, leukocytes can produce proteolytic enzymes that lead to tissue and organ damage, or differentiate into foam cells that lead to the atherosclerotic process[15].

Several animal models have shown that mice deficient in ICAM-1 are resistant to nephropathy in experimental models of diabetes, while treatment with anti-ICAM-1 monoclonal Ab prevents mononuclear cell infiltration into diabetic glomeruli[3].

Our group has shown that the levels of VCAM-1 correlate with the severity of albuminuria in diabetic hypertensive patients[15]. In addition, Seron *et al*[16] reported that VCAM-1 expression is increased in kidney biopsies from patients with DN, they also found a correlation between levels of VCAM-1 and numbers of infiltrating immune cells[18].

ADIPOKINES

Adiponectin and resistin were first described as adipocyte-secreted hormones (adipocytokines) that modulate insulin action. Both; hypoadiponectinemia and hyperresistinemia are associated with inflammation[19].

Hypoadiponectinemia has been reported as a risk factor for the development of albuminuria in mice[19], whereas in humans, resistin is mainly a monocyte-macrophage product. In humans hyperresistinemia promotes the expression of adhesion molecules[20], and is involved in the pathways that lead to albuminuria and renal damage[21].

WHICH INFLAMMATORY MOLECULE?

Certainly, inflammation is an important player in the pathogenesis of DN, However, because of multiple pathways that joint inflammation with diabetic complications, it looks unlikely that one single molecule be sufficient for the development of DN. It is also true that the blockade of the principal mediators could be useful in the prevention of this complication; several studies have been designed in order to indentify therapeutic targets.

The evidence suggest that TNF-α, MCP-1 and adhesion molecules have a prominent role in the development of DN, and all these mediators may be considered therapeutic targets for the prevention and treatment of DN, as we will discuss in the next section.

PERSPECTIVES

Microinflammation is the most important mechanism for development and progression of DN. Our knowledge related to signaling pathways involved in its pathogenesis has not been elucidated at all.

There are several pivotal mediators of inflammation, and their interactions are determinant in the process.

We have reviewed not only biological actions of these mediators, but also their possible therapeutic effects in experimental models.

The Smad family plays a very important role in inflammation and fibrosis in renal disease, its different actions among all molecular mediators leads to open several optional researches in DN.

A very interesting advanced is that if levels of Smad 7 could be restored in sick kidneys we could balance inflammatory responses in patients with renal diseases.

But not only Smad family could be a therapeutic option for DN patients, at this time it is very important take into a count that gene polymorphisms encoding several molecules in this patients have to be modified. Is in this way that investigations are aimed, looking to stop the progression of the disease, and not just for uncontrolled DM but also for other diseases involving the kidney.

Many options for interfering in transcription factors activation have been proposed, first blocking TF binding and second blocking TF pathways for activation. For these conditions there were used by both TF and experimental molecules.

Several studies are needed for interfering with signaling pathways not just for treatment of an abnormal condition as DN but also to prevent it.

Experimental studies have shown that inhibition of TNF-α (with the use of soluble TNF-α receptor fusion proteins, monoclonal antibodies or pentoxifylline) might be an efficacious treatment for renal disease secondary to diabetes mellitus, being pentoxifylline equivalent in efficacy and safety to captopril, and the addition of than drug to inhibitors of the renin-angiotensin system increases

their antiproteinuric effect[1,5].

Our group found that the reduction of urinary albumin excretion with the use of the fixed dose combination trandolapril-verapamil, depends not only from its antihypertensive effect, but also from its action on VCAM-1 adhesion molecules levels[22].

CONCLUSION

Inflammation plays an essential role in the development of DN, this participation involves increased chemokine production, infiltration of inflammatory cells to the kidney, pro-inflammatory cytokine production and tissue damage.

Several components of the diabetic milieu, as hyperglycemia, renin-angiotensin system and oxidative stress can activate the inflammatory process in the kidneys, which results in the infiltration of the organ by monocytes and lymphocytes, which secrete injurious molecules, such as proinflammatory cytokines and reactive oxygen species.

This leukocyte activity amplifies the inflammatory response and promotes cell injury and the development of fibrosis. Better understanding of the inflammatory response in diabetic kidneys is expected to identify novel anti-inflammatory strategies for the potential treatment of human DN.

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P- Reviewers: Bernieh B, Friedman EA **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Inflammation in diabetic kidney disease

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Author contributions: García-García PM, Getino-Melián MA and Domínguez-Pimentel V reviewed the literature and wrote the first draft of the manuscript; Navarro-González JF conceived the original idea, reviewed the first draft and revised versions, and finalized the manuscript; all authors approved the final version of the manuscript to be published.

Supported by Ministerio de Ciencia e Innovación (Instituto de Salud Carlos III-Fondo de Investigación Sanitaria), No. PI07/0870 and No. PI10/576; Ministerio de Sanidad y Política Social (Dirección General de Terapias Avanzadas y Trasplante), No. TRA-182; Sociedad Española de Nefrología y ACINEF; Research activity by Navarro-González JF is supported by Programa de Intensificación de la Actividad Investigadora, ISCIII/Canarias

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Received: October 4, 2013 Revised: February 24, 2014
Accepted: June 10, 2014
Published online: August 15, 2014

Abstract

Diabetes mellitus entails significant health problems worldwide. The pathogenesis of diabetes is multifactorial, resulting from interactions of both genetic and environmental factors that trigger a complex network of pathophysiological events, with metabolic and hemodynamic alterations. In this context, inflammation has emerged as a key pathophysiology mechanism. New pathogenic pathways will provide targets for prevention or future treatments. This review will focus on the implications of inflammation in diabetes mellitus, with special attention to inflammatory cytokines.

Key words: Diabetes; Diabetic nephropathy; Diabetic kidney disease; Inflammation; Cytokines; Oxidative stress

Core tip: Diabetic kidney disease is the main cause of renal insufficiency. This complication results from interactions of genetic and environmental factors that trigger a complex network of pathophysiological events. Inflammation has emerged as a key pathophysiology mechanism with important implications from a therapeutic perspective.

Original sources: García-García PM, Getino-Melián MA, Domínguez-Pimentel V, Navarro-González JF. Inflammation in diabetic kidney disease. *World J Diabetes* 2014; 5(4): 431-443 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/431.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.431

INFLAMMATION IN DIABETIC KIDNEY DISEASE

Diabetes mellitus (DM) is one of the most significant health problems worldwide. According to the projections, the number of adult diabetic patients will be higher than 430 million in 2030. Diabetic kidney disease (DKD) is one of the most prevalent complications, and is now the leading cause of end-stage renal disease (ESRD) in developed countries[1,2]. In the general population, ESRD rate increases due to the rise of diabetes mellitus. However, a recent study by Burrows *et al*[3] found that the incidence of ESRD in the diabetic population had shown a reduction, suggesting that the strategies for controlling DKD, including early diagnosis, adequate control targets and follow-up, early initiation of therapy, and the use of

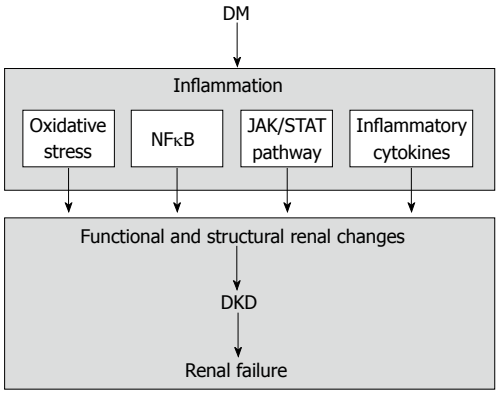

Figure 1 Schematic representation of inflammatory-mediated renal injury in diabetic kidney disease. DM: Diabetes mellitus; NFκB: Nuclear factor κB; DKD: Diabetic kidney disease; JAK/STAT: Janus kinase/signal transducers and activators of transcription.

effective renoprotective therapies, may be efficacious. However, it might be premature to state a real decline in ESRD in diabetes, since other reasons may be possible, such as the lack of enough time to develop ESRD in a large proportion of new diabetic subjects diagnosed in the last 20 years. In addition, the change of the diagnostic criteria for diabetes by the ADA in 1997, may have derived in the diagnosis of diabetes in a earlier stage of the disease, with a much less organ damage, and therefore, when diabetes have a more prolonged evolution, it is possible that this trends in the incidence of ESRD secondary to diabetes may reverse. Finally, another factor is the longer survival of diabetic patients, and thus, these subjects would have an increased risk of developing renal damage and ESRD.

Although kidney biopsy is required to definitively establish the diagnosis of DKD, in clinical practice this is unusual, since the careful screening of patients allow to identify people with DKD. The main criteria to diagnose DKD is the presence of an increased urinary albumin excretion (UAE), which is divided arbitrarily into microalbuminuria and macroalbuminuria, which is associated with an increased risk of decline in glomerular filtration rate (GFR) and a high risk of kidney failure.

DKD has been classically considered as the consequence from the interaction between hemodynamic and metabolic factors. However, renal damage is not completely explained by these factors. Current knowledge indicates that this represents only a partial view of a much more complex scenario. Clear evidence indicates that the pathogenesis of DKD is multifactorial, with the interaction of both genetic and environmental factors that trigger a complex network of pathophysiological events[4,5]. Clinical observations and epidemiological studies in different ethnic groups have indicated that there is familial aggregation of DKD. Although this information does not allow clearly establishing a model of transmission, diabetic nephropathy has been widely considered as a polygenic disease. There may be many genes, and each has a cumulative genetic effect and interacts with environmental factors in the development of DKD. The challenge in genetic studies of diabetic nephropathy is to dissect its genetic complexity. Researchers have searched for the genes involved in susceptibility, resistance or progression to DKD. The aim of genetic studies is to provide useful information for better understanding the pathogenesis and further developing novel therapeutic approach in this disease. Genome wide linkage analyses, candidate gene population association, family-based association and genome wide association studies have been used for the identification of the genes in DKD.

In this context, inflammation has become a cardinal pathophysiological mechanism in the development and progression of DKD. This review will focus on the implications of inflammation in DKD, with special attention to inflammatory cytokines.

INFLAMMATION IN DIABETES MELLITUS

Growing evidence indicates that pathogenesis of diabetes mellitus is widely related to the activation of the innate immune system and the presence of a chronic subclinical low-grade inflammatory state[6,7]. Many studies suggest that individuals who developed DM present characteristics of inflammation several years before the diagnosis of DM[8,9]. Population-based studies have shown that diverse inflammatory markers, such as cytokines, are strong predictors of the development of diabetes[10-12]. In addition, inflammatory cytokines have been involved in the pathogenesis of microvascular diabetic complications, including DKD[13-18].

DKD: AN INFLAMMATORY-BASED COMPLICATION

DM is associated with multiple deviations from normal homeostasis, including hemodynamic and metabolic alterations that produce the activation of diverse transduction pathways in the kidney. At the present time, inflammation is recognized as an important mechanism in the pathogenesis of this complication, through oxidative stress, transcription factors, including nuclear factor κB (NFκB), janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, and inflammatory cytokines[13,14] (Figure 1).

OXIDATIVE STRESS

There is solid experimental evidence of a key role for reactive oxygen species (ROS) and oxidative stress and their interplay with the renin-angiotensin-aldosterone system (RAAS) and inflammation, in the pathogenesis of DKD. There is a disproportionate production of ROS secondary to hyperglycemia by different renal cells[19-25]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that participates importantly in the regulation of the cellular antioxidant response[26,27]. Nrf2 appears to counteract renal damage in diabetes, possibly through inhibition of transforming growth factor-β1 (TGF-β1).

In both *in vitro* and *in vivo* experimental studies, Nrf2 ameliorated streptozotocin-induced renal damage. Nrf2(-/-) mice produced greater amounts of ROS and suffered more severe oxidative renal damage compared with wild type mice[28].

NFκB

NFκB is a transcription factor that controls the expression of genes involved in different processes, such as the immune response, cell differentiation and development, apoptosis, cycle progression, inflammation, and tumorigenesis. Importantly, this factor is activated by many stimuli related to DKD[29]. Many of the signalling molecules that produce the activation of NFκB may be potential targets for the inhibition of this factor, some of them acting within a network of signals leading to the activation of NFκB.

NFκB is continuously present in cells in an inactive state. In resting cells, NFκB dimers are cloistered by inhibitors of NFκB (IκBs), which prevents the translocation of NFκB to the nucleus. Triggering of the NFκB signalling cascade results in degradation of IκBs, allowing the liberation of NFκB, and thus, this factor translocates to the nucleus and induces transcription. IκB can be classified into several groups: classical IκB (IκBα, IκBβ and IκBε), NFκB precursors (p105 and p100) and nuclear IκB (IκBζ, Bcl-3 and IκBNS). All of them have a central ankyrin repeat domain (ARD), which permits the interaction with NFκB. The activation process of NFκB needs the phosphorylation of IκB, which results in polyubiquitination, a sign for destruction of the IκB by proteasome. The Ser/Thr-specific IκB kinases (IKKs) are the main points for the activation of NFκB. The IKK holocomplex incorporates IKKα or IKKβ, and the protein NEMO (IKKγ or FIP-3). IKK turning on occurs with phosphorylation of the activation loop Ser residues in the canonical MAP kinase kinase consensus motif SxxxS in the kinase domain. NEMO is crucial for the turning on of IKK since in cells without this protein, IKKα and IKKβ cannot be activated by any of the conventional NFκB activators. IKKβ is a key factor for turning on of the canonical NFκB pathway secondary to inflammation, whereas IKKα has a critical function in the non-canonical NFκB pathway through the phosphorylation of p100.

Different extracellular signals initiate the activation of NFκB. After entering the nucleus, this factor interacts with specific sequence motifs (κB sites) on their target genes, resulting in transcriptional turning on. The particular DNA-binding site characteristics of diverse NFκB dimers for a group of related κB sites, and the specific protein-protein binding at target promoters explain the specificity of NFκB signaling. In the majority of instances, turning on of NFκB is temporary and cyclical under the existence of a continuous inducer. This cyclical characteristic is secondary to recurrent destruction and production of IκB and the resulting turning on and inactivation of NFκB, respectively.

NFκB regulates a huge variety of target genes, including those coding for adhesion molecules, chemokines, inflammatory cytokines, nitric oxide synthase, and other molecules related to inflammation and proliferation, all of them involved in the pathogenesis of DKD[30]. NFκB is activated by a wide variety of stimuli[31] such as cytokines, oxygen radicals, inhaled particles, ultraviolet irradiation, bacterial or viral products, and metabolic abnormalities. High glucose may produce the activation of NFκB in diverse cells, including endothelial and vascular smooth muscle cells, and cells of the proximal tubule[32,33]. NFκB is central in the interplay among the different factors, molecules and pathways resulting in structural alterations and functional abnormalities observed in DKD, such as activation of the RAAS, advanced glycation end-products accumulation, and NADPH-dependent oxidative stress[34]. In experimental models of DKD, it has been established the activation of NFκB in the renal cortical tissue[35]. Moreover, in human DKD, proteinuria itself, is an important activator of NFκB and it's an important pro-inflammatory stimulus for tubular cells. Chemoattractants and adhesive molecules for inflammatory cells are upregulated by excess ultrafiltered protein load of proximal tubular cells *via* activation of NFκB-dependent and NFκB-independent pathways[36].

NFκB represents a central factor in inflammation, with the generation of intrincated regulatory circuits that include a huge variety of cellular mediators, such as adhesion molecules, intracellular second messengers, microRNA, growth and transcription factors, and cytokines. NFκB system is critical for the flow of biological messages from DNA information to protein synthesis. In addition, these elements have important pathogenic and pathophysiologic roles in human disease, including DKD.

JAK/STAT PATHWAY

In animal models and in clinical studies in DKD, it has been demonstrated the enhanced activation of JAK/STAT pathway in the glomeruli and tubulointerstitial cells. The JAK proteins are intracellular, non receptor tyrosine kinases that transduce cytokine-mediated signals. Secondary to the binding of the ligand to the cytokine receptor, the JAK proteins associated with the intracellular domain of the receptor, phosphorylate and activate each other. The autophosphorylation of the JAK proteins induces a conformational modification, allowing the transduction of the intracellular signal by further phosphorylating and activating the STAT transcription factors. The activated STAT molecules dissociated from the receptor and form dimers and translocate to the cell nucleus, where they activate many target genes. The JAK/STAT signaling route is a major connecting system between the receptors located at the cell surface and the transcriptional events occurring within the cell nucleus.

It has been demonstrated the great importance of the JAK/STAT pathway in the pathogenesis of DKD through its participation in several processes, such as the

hypertrophy of mesangial cells induced by angiotensin Ⅱ (Ang Ⅱ), and the synthesis of TGF-β, collagen Ⅳ and fibronectin. In addition, the high levels of glucose stimulate the production of ROS within the cells, which in turn activates the JAK/STAT pathway.

Although there are several types of JAK proteins, the one primarily studied in renal and vascular tissue is JAK2[37]. Experimental studies in animal models of diabetic nephropathy have showed that hyperglycemia is able to turning on the JAK2/STAT pathway in renal cells[38-42]. Moreover, clinical studies in patients with early of advanced stages of DKD have showed an increased expression of JAK/STAT mRNAs and JAK2 protein in the glomerular and tubulointerstitial compartment, with an inverse correlation between JAK2 mRNA levels and estimated GFR in these patients[43].

The intimate mechanism by which hyperglycemia promotes JAK2 activation has been related to the interaction between JAK2 and ROS caused by high glucose. ROS enhance the activity of JAK2, whereas the use of an inhibitor of ROS formation (diphenylene iodonium) resulted in a marked inhibition of Ang Ⅱ-induced activation of JAK2. These facts reveal that ROS act as an intracellular activator of the JAK-STAT pathway, and that ROS also act as a second messenger for the regulation of JAK2 activation by Ang Ⅱ. One of the leading causes of the increased JAK2 tyrosine phosphorylation is the alteration of tyrosine phosphatases (SHP-1 and SHP-2). SHP-1 phosphorylation is abolished under hyperglycemia, whereas SHP-2 phosphorylation is increased under basal and Ang Ⅱ stimulation, suggesting that JAK2 sustained activation under hyperglycemia is partly due to decreased SHP-1 and increased SHP-2 phosphorylation. In addition, these effects are due to hyperglycemia and not to hyperosmolarity, since no alterations in the tyrosine phosphorylation of both SHP-1 and SHP-2 have been observed under conditions with elevated osmolarity without hyperglycemia[38-41].

INFLAMMATORY CYTOKINES

Cytokines are low molecular weight polypeptides with autocrine, paracrine and juxtacrine effects, and very complex activities. The classic function of cytokines is related to the regulation of the inflammatory process, but they are also crucial effectors of the immune system. Cytokines often have multiple target cells and multiple pleiotropic actions, and thus a particular cytokine may activate diverse reactions based on the type of cell, the time of action, and the situation and ambience. Moreover, cytokines may share receptor subunits and intracellular signalling pathways, and they can act synergistically in many contexts[44].

The first studies suggesting that inflammatory cytokines were engaged in the pathogenesis of DKD were published more than 20 years ago by Hasegawa *et al*[45,46]. The authors reported that glomerular basement membranes (GBM) obtained from rats after the induction of diabetes, were able to induce the production of significantly higher quantity of the inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin 1 (IL-1) when were incubated with peritoneal macrophages, as compared with the production of those cytokines when the macropages were cultured with membranes from normal rats. Later works showed that all types of resident renal cells, as well as infiltrating cells (monocytes, macrophages and lymphocytes) are able to synthesize proinflammatory cytokines[47,48]. Nowadays, the results of numerous studies support the notion that cytokines play a transcendent role in the pathogenesis of microvascular complications of DM[13,49,50]. The renal effects of cytokines in DKD are associated with different actions, including intrarenal hemodynamic alterations, modifications of the renal structure with changes in extracellular matrix and basement membranes, abnormalities in the expression of diverse molecules, cellular necrosis and apoptosis, modification in the permeability of glomerular endothelium, and increment in the production of ROS[50-54].

IL-1

In experimental models of DKD, renal expression of IL-1 is elevated[55,56], which has been associated with changes in the expression of molecules related to chemotaxis and cellular adhesion. Specifically, IL-1 augments the production of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 by different renal cells, including endothelial, mesangial and tubular epithelial cells. In addition, IL-1 also stimulates the expression of endothelial-leukocyte adhesion molecule 1[57,58].

IL-1 produces abnormalities of intraglomerular hemodynamics. These effects are secondary to modifications in the synthesis of prostaglandins by mesangial cells. Experimental *in vitro* studies have shown that glomerular mesangial cells incubated with recombinant human IL-1 are stimulated to produce prostaglandin E2 and delivery phospholipase A2[51]. Futhermore, these cells present an increased secretion of prostaglandin E2 in response to Ang Ⅱ[52], whereas the permeability of vascular endothelial cells is enhanced[59]. Finally, this cytokine raises the production of hyaluronan by epithelial cells of renal proximal tubule[60], which has been related with the development of hypercellularity in experimental models of diabetes[61].

IL-6

Clinical studies have shown that IL-6 levels are significantly higher in patients with DKD in comparison with DM patients without nephropathy[62]. In addition, the histopathological analysis of human renal samples by immunohistochemistry has demonstrated an increased expression of mRNA encoding IL-6 in cells infiltrating the mesangium, interstitium and tubules, with a positive relationship with the severity of mesangial expansion[63]. Other functional and structural abnormalities related to DKD and progression of renal damage have been associ-

ated with IL-6, including abnormalities in the permeability of glomerular endothelium, expansion of mesangial cells and enhanced expression of fibronectin[54] and increase in the thickness of the GBM[64,65]. Our experimental studies have demonstrated an increase in the mRNA levels of IL-6 in the renal cortex of diabetic rats, which is positively associated with the urinary concentration of this cytokine[56]. In addition, in animal models of diabetes, wet kidney weight, a marker of renal hypertrophy and an early phenomenon in kidney involvement in DM[66], has been reported to be enhanced, which was related to mRNA gene expression levels and urine concentration of this cytokine[56].

IL-6 signals through a cell surface receptor, which is formed by the ligand-binding IL-6 receptor (IL-6R)-α chain (CD126) and the signal-transducing component CD130, also called gp130. In addition to the membrane form of the IL-6R, there is a soluble form which is produced by cleavage of the membrane-bound form. These soluble form of the IL-6R comes to the circulation and is able to control the activity of this cytokine. Regarding this regulatory process, it is important to differentiate the actions of soluble CD126 and CD130. In plasma, soluble CD126 binds to IL-6 and results in the increase of the complex half-life, amplifying the bio-activity of this cytokine to tissues that express the membrane form of CD130. On the contrary, soluble form of CD130 in the circulation functions as an IL-6 antagonist. Recent studies have shown that the soluble form of the IL-6R is closely implicated in the evolution from the initial to the final stages of the inflammatory reaction. IL-6 has many biological properties, including the activation of the STAT3 transcription factor, and the induction of the expression of adhesion molecules and other inflammatory cytokines.

IL-18

IL-18, a potent inflammatory cytokine that belongs to the IL-1 superfamily[67,68], is implicated in different actions, including the release of interferon (IFN)-γ[69] (which stimulates functional chemokine receptor expression in human mesangial cells)[70], the synthesis of other molecules involved in the inflammatory reaction, such as IL-1 and TNF-α, the increase in the expression of ICAM-1, and the apoptotic process of endothelial cells[71-73]. Tubular renal cells show an increase in the expression of IL-18 in patients with DKD[74], which has been related to the triggering of mitogen-activated protein kinase (MAPK) pathways secondary to the action of TGF-β[75]. Many other cells may also produce this cytokine, such as infiltrating monocytes, macrophages and T cells[67,68]. High levels of IL-18 has been found in serum and urine of patients with DKD, with an independent relationship with UAE[76-78]. In addition, serum IL-18 levels are associated with the urine concentration of β-2 microglobulin, a low-weight protein that is used as a marker of tubular dysfunction[77]. In a recent longitudinal study in patients with type 2 diabetes, serum and urinary levels of IL-18 were direct and independently associated with UAE. In addition, the concentrations of this cytokine in serum and urine were also significantly associated with changes in albuminuria during the evolution of the study[77].

TNF-α

TNF-α is a cytokine with prominent proinflammatory effects. It is mainly produced by monocytes, macrophages and T cells, but also intrinsic kidney cells[47,79-81]. TNF-α exists in the cells as a precursor of the active form. This precursor is transformed in the active form through the action of the TNF-α-converting enzyme[82]. There are two specific TNF-α receptors: the TNF-α receptor 1 (TNFR1), an epithelial-cell receptor also named p55, and the TNFR2, which is an myeloid-cell receptor (p75). The exact roles of the receptors are not yet completely understood and may differ depending on the organ type[83]. While TNFR1 modulates the immune response (IL-6 synthesis) and apoptosis (apoptotic signaling kinase 1 and NFκB of mesangial cells), TNFR2 has been recognized as one of the proinflammatory mediators in glomerulonephritis[84,85]. After binding to these receptors, the intracellular transduction cascade is activated, leading to the final biological actions of this cytokine[86], with a potential role in the pathogenesis of DKD. Experimental studies in animal models of diabetes have showed that TNF-α levels and mRNA encoding TNF-α are enhanced in renal glomeruli and tubules[47,56,80,87-89].

TNF-α may cause direct cytotoxicity to renal cells, inducing direct renal injury[90], apoptosis and necrotic cell death[91,92]. It can also produce alterations of intraglomerular blood flow and reduction of glomerular filtration as consequence of the disequilibrium between factors promoting vasoconstriction and vasodilation[93], in addition to changes in the permeability of endothelial cells. Other actions of this cytokine are the modification in the location of molecules involved in the adhesion process among cells, such as the endothelial-cadherincatenin complexes, as well as the alteration of normal endothelial permeability due to alterations of cellular junctions secondary to the lack of F-actin stress fibers[94]. In addition, TNF-α is able to directly induce the formation of ROS by renal cells[95]. Experimental researches has shown that TNF-α induces the activation of NADPH oxidase in isolated rat glomeruli through the activation of the intracellular pathways protein kinase C/phosphatidylinositol-3 kinase and MAPK[96]. Thus, TNF-α prompts local ROS production, independent of hemodynamic mechanisms, resulting in alterations of the glomerular capillary wall and consequently increased albumin permeability[53].

An increase in renal size (kidney hypertrophy) and glomerular filtration rate (hyperfiltration) are early and relevant findings of DKD, which are significantly related to TNF-α[88,89]. *In vitro* studies demonstrated that TNF-α stimulates the solute uptake in proximal tubular cells secondary to the activation of sodium-dependent cotransporters[97], whereas *in vivo* studies in diabetic rats found an enhanced urinary excretion of TNF-α excretion, which was related to sodium retention and renal hypertrophy.

All these effects could be blocked by the use of a soluble TNF-α receptor fusion protein[89,97]. In the renal distal tubule TNF-α activates the epithelial sodium channel resulting in an increased reabsorption of sodium, which can be abrogated by blockers of this renal channel, such as amiloride, and inhibitors of extracellular signal related protein kinase. The increment in renal sodium reabsorption might induce the expression of TFG-β, with the development of renal hypertrophy[98].

Expression mRNA levels in the renal cortex and urinary TNF-α excretion show a positive and independent correlation with albuminuria[56,87]. Moreover, microdialysis studies showed that the concentration of TNF-α in the kidney interstitial fluid is elevated, as well as in the urine, with no data of cellular renal infiltration. These findings are observed previously to the detection of an increase in UAE. In addition, there is an elevation in the levels of TNF-α in urine after the increase in UAE, which suggest that the rise of albuminuria has a stimulatory effect in the production of TNF-α by the kidney[99]. These findings support the intimate relationship between proteinuria and inflammation. Current data indicates that proteinuria per se is an important factor in the development of tubulointerstitial damage, but also by the capacity of activate an inflammatory cellular response *via* chemoattractants, adhesive molecules and proinflammatory cytokines. These changes lead to the renal infiltration by blood circulating cells, with the subsequent damage to renal cells, damage of tubular and interstitial structures, and finally, to the development of renal fibrosis and scarring[100].

Finally, many clinical studies in patients with DKD have reported that the serum and urinary concentrations of TNF-α are elevated as compared with non-diabetic individuals or with diabetic subjects and kidneys, and that these concentrations increase concomitantly with the progression of DKD. These findings indicate a potential relationship between the elevated levels of this inflammatory cytokine and the development and progression of renal injury in DM[76,101,102].

In addition to TNF-α, also TNF-α receptors have been related to DKD. In an observational study in type 1 diabetic patients, the serum levels of TNFR1 and TNFR2 were linked with renal function with independence of other variables, such as albuminuria, supporting the important participation of this cytokine in DKD[103]. In addition, this involvement has also been found in type 2 DM (T2DM). Thus, after more than 10 years of follow-up, the Nurses' Health Study showed that increased concentrations of the soluble TNFR2 were a powerful predictor of the loss of renal function in these patients[104].

Finally, are also important the findings derived from studies focused on another cytokine within the TNF superfamily, the TNF-α-related apoptosis-inducing ligand (TRAIL). TRAIL participates in diverse cellular processes, including apoptosis, cell expansion and maturity[105]. Clinical studies in patients with diabetes have shown that the renal expression of this cytokine is enhanced, and more importantly, the grade of expression is directly related with the seriousness of kidney injury[106]. Regarding the cell types that express TRAIL, immunohistochemistry studies demonstrated that the renal expression of this cytokine was maximal in tubular epithelial cells. However, it is important to highlight that the expression of TRAIL has been also observed in podocytes[106,107]. It has been suggested the participation of TRAIL in the pathogenesis of DKD based on the finding that the magnitude of renal tissue staining for this cytokine was directly associated with the grade of tubulointerstitial inflammation, scarring and degeneration.

INFLAMMATION IN DKD: A THERAPEUTIC OPPORTUNITY

Established therapeutic strategies for prevention and treatment of DKD focus on blood pressure and glucose control, RAAS blockade and anti-thrombotic/-inflammatory treatment with aspirin. However, these therapies are insufficient[108] and new approaches are required[109].

Oxidative stress

In experimental models, the administration of different antioxidant drugs (tempol, thiol, kallistatin)[110-112] improved oxidative stress-induced renal injury, decreasing albuminuria and fibrosis. Triterpenoids, synthetic analogues of oleanolic acid with potent anti-inflammatory and antioxidant properties, activate the ARE-Keap1-Nrf2 pathway.

The renoprotective action of bardoxolone methyl, a triterpenoid that reduces oxidative stress and inflammation through Nrf2 activation and inhibition of NFκB, has been recently explored in humans. A large multicenter double-blind, randomized trial (BEAM study), including 227 patients with moderate-severe CKD and T2DM, showed that administration of bardoxolone was associated with significantly improvement of GFR at 24 wk, but some adverse events were found (mild reversible increase of albuminuria, decreased serum magnesium, muscle spasms, nausea and loss of body weight)[113]. Later, the BEACON trial, a multinational, multicentric and double-blind randomized, placebo-controlled Phase 3 trial, was designed to determine whether bardoxolone would have beneficial effects on the progression of renal injury and the hazard of ESRD in subjects with T2DM and severe stages of renal disease. Regrettably, the increased risk of heart failure and cardiovascular events observed in the bardoxolone arm of the BEACON study led to the premature ending of this trial[114].

The most commonly reported serious adverse event in the bardoxolone group was heart failure. The mechanism linking bardoxolone methyl to heart failure is unknown, although some aspects deserve consideration. Firstly, body weight declined significantly in the bardoxolone methyl group, which may suggest a situation of hemodilution secondary to fluid retention, since a reduction in the serum albumin and hemoglobin concentrations was observed. Secondly, it was observed an increase in

blood pressure in the bardoxolone arm, which might result in an elevation of cardiac afterload. This fact, together with the increase in heart preload secondary to fluid retention, combined with a rise in heart rate, result in a situation likely to trigger heart failure. This hypothesis is congruent with the increase in the concentration of B-type natriuretic peptide with bardoxolone methyl, which may reflect an elevated left ventricular wall stress.

NFκB

The renoprotective effects conferred by blockade of RAAS, provides pleiotropic and anti-inflamatory issues through the suppression of NFκB-dependent pathways, beyond the control of blood pressure and proteinuria[115]. In addition, the beneficial effects on the kidney showed by other drugs, such as thiazolidinediones, have been also associated to a suppressive effect on the activation of this transcription factor[116,117]. In addition, recent experimental studies indicates that suppression of NFκB activation by various agents, such as 1,25-dihydroxyvitamin D3[118], cilostazol[119], and curcumin[120], could lead to amelioration of DKD, suggesting the importance of NFκB as a therapeutic target of DKD.

JAK/STAT pathway

Studies in experimental animal models of DKD have reported that the use of AG490, a specific tyrosine kinase inhibitor of JAK2, was able to abrogate the elevation of systolic blood pressure[121] and the increase of UAE[122]. On the other hand, recent studies have highlighted the role of suppressors of cytokine signaling (SOCS) proteins, a group of molecules that bind and interfere with initiating JAK proteins, and act as intracellular negative regulators of JAK/STAT activation in DKD[37]. Ortiz-Muñoz *et al*[123] demonstrated that high concentrations of glucose were associated *in vitro* with activated JAK/STAT/SOCS in human mesangial and tubular cells. Overexpression of SOCS reversed the glucose-induced activation of this pathway, expression of STAT-dependent genes and cell proliferation. On the other hand, the inoculation of recombinant SOCS1 and SOCS3 adenovirus to diabetic rats resulted in an improvement of renal function at 7 wk, and renal lesions such as mesangial expansion, fibrosis or influx of macrophages were also reduced. However, further research into JAK inhibitors, SOCS expression or SOCS mimetics is required, given the critical immunomodulatory role of this pathway, with possible adverse effects[37].

Inflammatory cytokines

Experimental works using animal models of both types of DM have revealed probable benefits from the use of immunosuppressive drugs. Mycophenolate mofetil (MMF), an immunosuppressive agent with anti-inflammatory properties, was able to avoid the initiation and progression of glomerular damage and albuminuria in rats with streptozotocin-induced diabetes[124]. Subsequent works demonstrated that MMF produced a marked reduction of proteinuria, as well as the amelioration of both renal glomerular and tubulointerstitial scarring[125]. All these renoprotective effects did not have any relationship with beneficial changes of hemodynamic or metabolic determinants, suggesting that the benefits probably resulted from its immunosuppressive and anti-inflammatory actions. Thus, it was demonstrated that MMF is able to reduce glomerular and tubulointerstitial inflammatory cell infiltration[126] and abrogate different processes related to the action of TNF-α, such as the expression of ICAM1, the adhesion of neutrophils to the endothelium, as well as the production and discharge of inflammatory cytokines (IL-6 and TNF-α)[127-129]. Despite these promising experimental results, immunosuppressive treatments actually are not a current clinical therapeutic option in patients with DKD.

Modulation of inflammatory cytokines, mainly TNF-α, has been evaluated in experimental works, as well as in studies with diabetic patients. In experimental studies, the use of etanercept, a recombinant human soluble TNF-α receptor, was associated with the reduction of the urinary excretion of this cytokine and the avoidance of initial kidney structural injury and renal hypertrophy in experimental models of DKD[88]. Similarly, the use of the monoclonal anti-TNF-α antibody infliximab on rats with DKD led to a significant reduction in the urine excretion of TNF-α and albuminuria[130]. At the present time, the use of soluble TNF-α receptors or monoclonal antibodies as therapy for DKD have been not tested in clinical trials. However, pentoxifylline (PTF), a drug used in the treatment of peripheral vascular disease, possesses modulating effects on TNF-α, with significant anti-inflammatory properties that has potential clinical applications as a therapy for DKD.

PTF, a methylxanthine derived with non-specific phosphodiesterase activity, possess significant anti-inflammatory properties: this drug is able to abrogate the transcription of the *TNF-α* gene and hamper the augmentation of TNF-α mRNA[131,132], regulate IL-1, IL-6 and IFN-γ, and lessen diverse cell actions related to inflammation, such as activation, adherence and phagocytosis[133,134]. PTF is able to reduce the generation of profibrotic factors (fibronectin and TGF-β) in human mesangial cells caused by elevated glucose levels, and also it protects these cells from the harmful effects of angiotensin Ⅱ on matrix proteins[135]. Furthermore, in animal models of DKD, PTF significantly decreased the width of the GMB, the plastering of podocyte foot processes, and the disappearance of the fenestrations of glomerular endothelium[136]. In addition, PTF prevents the increased renal expression of the inflammatory cytokines TNF-α, IL-1 and IL-6 secondary to diabetes, resulting in a reduction of UAE, the urinary concentration of these cytokines, as well as a decrease of renal hypertrophy and sodium retention[56,87,88].

Beyond the results from experimental works, a number of clinical studies have showed that PTF is effective to reduce albuminuria and has potential beneficial effects on renal function in diabetic patients[137-143]. The antipro-

teinuric action of PTF has been straightly associated with its anti-TNF-α activity. This effect has been demonstrated to affect molecules with a high and a low molecular weight, such as IgG, ceruloplasmin, transferrin, albumin, and α 1-antitrypsine, lysozyme and β2-microglobulin, respectively[144]. The reduction of proteinuria after PTF administration has been confirmed in various prospective, controlled, randomized clinical studies[144-146]. Furthermore, PTF has showed beneficial effects on the urinary excretion of markers of tubular damage, such as N-acetylglucosaminidase[145]. The effectiveness of PTF to reduce urinary protein excretion has been compared with that of angiotensin-converting enzyme inhibitors (ACEI) in T2DM, and the results reveal that PTF is similar to captopril[144,145]. Moreover, the use of PTF on top of blockade of the RAAS with ACEI or angiotensin Ⅱ receptor blockers, provide a supplementary and synergistic decrease of albuminuria[147,148], an effect not related to blood pressure and metabolic control, but positive and directly related with a lowering in the urinary concentration of TNF-α[147].

The capacity of PTF to reduce UAE in subjects with DKD has been confirmed by a recent meta-analysis, which highlighted that the anti-inflammatory properties of this drug, with a decrease in the generation of proinflammatory cytokines, was the main potential mechanism to explain its antiproteinuric effect[149]. A prospective, randomized clinical trial is now ongoing to evaluate the effects of PTF on the renal function of patients with DKD[150], and new definitive trials (multicentre, adequately powered, prospective, placebo controlled) are needed to give definitive evidence for the use of PTF as a real option for the treatment of DKD.

CONCLUSION

Diabetes mellitus is a major global health problem. DKD is one of the most important complications and constitutes a challenge for physicians. Conventional treatments provide incomplete protection for the development of renal failure. Therefore, new approaches and therapeutic targets are needed. Based on the results of recent studies, nowadays inflammation is acknowledged as a key factor in the development and progression of DKD. Future therapies will focus on modulation of inflammatory pathways, including targets such as inflammatory cytokines, oxidative stress, JAK/STAT pathway, or NFκB. In addition, further research is needed to understand how inflammatory pathways interact with other pathogenic factors in the context of diabetes.

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P- Reviewer: Cui WP, Ozdemir S, Theilade S **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diabetes treatment in patients with renal disease: Is the landscape clear enough?

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Received: November 30, 2013 Revised: July 9, 2014
Accepted: July 18, 2014
Published online: October 15, 2014

Abstract

Diabetes is the most important risk factors for chronic kidney disease (CKD). The risk of CKD attributable to diabetes continues to rise worldwide. Diabetic patients with CKD need complicated treatment for their metabolic disorders as well as for related comorbidities. They have to treat, often intensively, hypertension, dyslipidaemia, bone disease, anaemia, and frequently established cardiovascular disease. The treatment of hypoglycaemia in diabetic persons with CKD must tie their individual goals of glycaemia (usually less tight glycaemic control) and knowledge on the pharmacokinetics and pharmacodynamics of drugs available to a person with kidney disease. The problem is complicated from the fact that in many efficacy studies patients with CKD are excluded so data of safety and efficacy for these patients are missing. This results in fear of use by lack of evidence. Metformin is globally accepted as the first choice in practically all therapeutic algorithms for diabetic subjects. The advantages of metformin are low risk of hypoglycaemia, modest weight loss, effectiveness and low cost. Data of UKPDS indicate that treatment based on metformin results in less total as well cardiovascular mortality. Metformin remains the drug of choice for patients with diabetes and CKD provided that their estimate Glomerular Filtration Rate (eGFR) remains above 30 mL/min per square meter. For diabetic patients with eGFR between 30-60 mL/min per square meter more frequent monitoring of renal function and dose reduction of metformin is needed. The use of sulfonylureas, glinides and insulin carry a higher risk of hypoglycemia in these patients and must be very careful. Lower doses and slower titration of the dose is needed. Is better to avoid sulfonylureas with active hepatic metabolites, which are renally excreted. Very useful drugs for this group of patients emerge dipeptidyl peptidase 4 inhibitors. These drugs do not cause hypoglycemia and most of them (linagliptin is an exception) require dose reduction in various stages of renal disease.

Key words: Chronic kidney disease; Diabetes; Antidiabetic drugs; Metformin; Dipeptidyl peptidase 4 inhibitors; Therapeutic algorithm

Core tip: Chronic kidney disease (CKD) is very often among diabetic persons. In every day clinical practice doctors worldwide have to deal with these patients and help them to achieve their metabolic goals. Despite this, many studies of antidiabetic drugs have excluded people with CKD. So, we lack solid evidence on the effectiveness and safety of these drugs. In this review I propose therapeutic algorithms for diabetic patients in different stages of CKD and clarify some questions about the use of popular antidiabetic drugs as metformin and sulfonylureas.

Original sources: Ioannidis I. Diabetes treatment in patients with renal disease: Is the landscape clear enough? *World J Diabetes* 2014; 5(5): 651-658 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/651.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.651

INTRODUCTION

Chronic kidney disease (CKD) affects million of people

worldwide. CKD is becoming a major problem for public health as it leads to increased morbidity and mortality. Patients with end stage chronic kidney disease often need kidney transplantation[1].

The prevalence of chronic kidney disease to the estimated 11% of the United States population. Patients with chronic kidney disease have an increased risk of cardiovascular disease and progression of renal disease in end-stage renal failure. End stage renal failure leads to dialysis or transplantation[2,3].

Diabetes is the most important risk factors for CKD. The risk of CKD attributable to diabetes continues to rise worldwide.

The National Kidney Foundation and the American Heart Association have recently issued guidelines for the management of cardiovascular risk in people with kidney disease by stating emphatically that these individuals are at very high cardiovascular risk.

For diabetic patients with chronic kidney disease, the risk of cardiovascular disease is even higher classifying these individuals in the highest risk group for cardiovascular disease. Diabetic subjects with microalbuminuria have increased risk (2x) of cardiovascular disease than those with normoalbuminuria. Proteinuria and decreased Glomerular Filtration Rate (GFR) contribute synergistically to increase cardiovascular risk. Most diabetic patients with CKD stage 3 will suffer a serious cardiovascular event, possibly fatal before their chronic kidney disease progress to end stage kidney failure.

Diabetic patients need complicated treatment for their metabolic problems as well as for related comorbidities. They have to treat, often intensively, hypertension, dyslipidaemia, bone disease, anaemia, and frequently established cardiovascular disease (CVD). Thus, the problem for the appropriate selection of antidiabetic treatment for patients with diabetes and CKD is usual in every day clinical practice[4,5].

DIABETES TREATMENT: DIFFERENT GOALS AND DIFFERENT DRUGS

Recent guidelines for the treatment of diabetes (ADA, EASD 2012) propose personalization of glycaemic goals. For the majority of diabetic patients the appropriate goal is a haemoglobin A1c (HbA1c) < 7% but for patients with severe comorbidities a goal between 7% and 8% is acceptable. Diabetic subjects with CKD usually belong to this group.

The glycated HbA1c is the most popular and well-accepted biological marker for the assessment of long-term glycaemic control. This also applies to patients with diabetes and renal disease. However, the method has significant limitations in these patients. The measurement is influenced by both renal function and complications of chronic kidney disease such as haemolysis, iron deficiency and metabolic acidosis.

In most cases diabetic subjects with chronic kidney disease must rely more on self-monitoring of blood glucose with usual glucose meters. Patients with diabetes and CKD have usually already established CVD. These patients are also in greater risk of hypoglycaemia. We know from physiology that normal renal function conveys a 30% of neoglycogenesis, which is necessary to avoid hypoglycaemia especially in prolonged fasting periods[6].

Many diabetics with uraemia have also nutritional problems and some times cachexia. The use of insulin as well as of sulfonylureas or glinides (short acting secretagogues) leads to increased rate of hypoglycaemia in this group of patients[7,8].

On the other hand, many drugs have renal metabolism and their metabolites are usually active prolonging their time of action. The use of antidiabetic drugs, especially the new classes, is conflicted. The major problem is that in many efficacy studies patients with CKD are excluded so data of safety and efficacy for these patients are missing. This results in fear of use by lack of evidence[9].

Nevertheless, pharmacokinetics and pharmacodynamics data for many new drugs help us to understand the potential risks and benefits for these subjects. Even if these basic data are reassuring the clinical point remains critical: We cannot use new drugs based only on these evidence! We need results form efficacy studies and then approval from FDA and EMEA[10].

Finally, the use of antidiabetic drugs is more complicated in these patients because many people with kidney disease are often elderly, and have long lasting disease and significant co-morbidities. These people take many drugs and they have high risk of drug interactions.

ESTIMATION OF RENAL FUNCTION IN DIABETIC PATIENTS

For all diabetic subjects we have to estimate their renal function. 1st step: Serum creatinine/annually (or every 3-4 mo in selected patients); 2nd step: Based on serum creatinine we estimate GFR (eGFR). eGFR is usually based on patient characteristics (as age, sex and race) as well as serum creatinine levels. The most popular method of assessment of renal

$$\text{MDRD: GFR} = 175 \times \text{SerumCr}^{-1.154} \times \text{age}^{-0.203}$$

[× 1.212 (if patient is black) × 0.742 (if female)]

function with the greater precision is the Modification of Diet in Renal Disease (MDRD) equation. This equation is based on data of MDRD Study. This equation (MDRD) is especially accurate in GFR < 60 mL/min.

For higher GFR another equation can also be used: Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) method based on data of CKD-EPI.

$$\text{CKD-EPI: GFR} = 141 \times \min(\text{Scr}/\kappa, 1)^{\alpha} \times \max(\text{Scr}/\kappa, 1)^{-1.209} \times 0.993^{\text{Age}} \times 1.018$$

(if female) × 1.159 (if black)

Where Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1.

Usually we use friendly calculators to estimate GFR.

Many programs are also free available for smartphones.

The classical formula of Cockcroft-Gault is not used anymore because it overestimates GFR. (Body weight in the formula must be lean weight and not total weight).

METFORMIN

Metformin is globally accepted as the first choice in practically all therapeutic algorithms for diabetic subjects. The advantages of metformin are low risk of hypoglycaemia, modest weight loss, effectiveness and low cost. Data of UKPDS indicate that treatment based on metformin results in less total as well cardiovascular mortality.

Many diabetologists as well as practitioners are fear to use metformin in patients with renal problems even if they have only albuminuria. There is a lot of confusion about the real restriction of its use in patients with CKD[11].

Metformin is slowly absorbed when administered orally. The bioavailability of the drug is low (50%-60%).

Metformin achieves a maximum plasma concentration one to three hours after ingestion, if taken in the form of immediate release or in 4-8 h with the extended release form. Metformin is not connected with albumin or any other protein in plasma. This results in a high volume of distribution up to 1000 even after the first dose[12].

In patients with moderate and severe CKD Cmax is increased 173% and 390%, respectively, compared with patients with normal renal function.

In normal pH metformin remains as hydrophilic cation. Less than 0.01% of the drug is unionized in blood. Lipid solubility of metformin is low. So, metformin can not diffuses through cell membranes. Phenformin, another member of antidiabetic drug class biguanides, which is no longer in the market, is more lipophilic than metformin due to different side chain. Metformin is not metabolized in the liver. Metformin is actively excreted by the urinary tube and found unchanged in the urine. After 24 h, if renal function is normal, metformin is not detected in the blood after administration of a single dose. The half-life of metformin is plasma is about 6 h[13].

The absorption of metformin in the intestine is mediated by a transporter known as plasma membrane monoamine transporter. Several metformin transporters are implicated in its intestinal absorption as well as in its hepatic uptake and renal excretion. These transporters are either Organic Cation Transporters (OCTs) or multidrug and toxin extrusion proteins (MATEs).

The kidneys also actively excrete metformin. Metformin enters renal cells of the renal tubule from circulation. This procedure takes place on the basolateral membrane of the cells and is mediated by OCT2.

Then, metformin is excreted into the lumen. This excretion is facilitated by MATE (1 and 2-K). These extrusion proteins are located in the apical membrane of renal proximal tubule cells.

Metformin is also reabsorbed in renal tubules and this action is mediated by OCT1, which is located also in proximal and distal tubules.

The molecular mechanisms underlying metformin action appear to be complex. Metformin entries into the hepatic cell and facilitate phosphorylation and activation of AMP-activated protein kinase (AMPK). Activation of this key-kinase (energy status sensor) lead to many effects related to metabolism of glucose and lipids. Metformin inhibits hepatic neoglycogenesis also in a direct manner. Metformin inhibits complex I of the mitochondrial respiratory chain. This inhibition, leads to an incretion of AMP:ATP ratio, which activate AMPK. This inhibition leads also to increased anaerobic metabolism of glucose in cytoplasm and the production of lactic acid. Thus, metformin is related with increased risk of lactic acidosis when renal elimination of lactic acid is decreased (renal disease, reduced GFR) or hepatic function is severely damaged (lactic acid is used in hepatocytes to produce glucose-neoglycogenesis-). The risk of lactic acidosis is also increased in patients with tissue hypoxia (shock, severe heart failure, sepsis, surgery related hypotension, *etc.*)[14].

Risk of lactic acidosis was greater with phenformin because it's a more potent inhibitor of mitochondrial respiration. Phenformin has hepatic metabolism with an inactive metabolite. The enzyme CYP2D6 metabolizes phenformin into an inactive metabolite. A small ratio of patients (about 2.8%) has a polymorphism of the enzyme that makes them poor metabolizers. In these patients the risk of lactic acid is even greater (due to higher levels of phenformin).

Nevertheless, analysis of data from may trials (347 comparative trials and cohort studies) from Cochrane Database systematic review in 2010, showed no cases of lactic acidosis in 70490 patient-years of metformin.

Statistical analysis of these data suggested that the upper limit for the incidence of lactic acidosis per 100000 patient-years was 4.3 cases (lower than 5.4 cases in the non-metformin group).

In this analysis also, levels of lactic acid seems to be no different in the two groups.

In most studies however lactic acidosis was not a pre specified end point and there were no data about lactic acid levels.

In the Table 1 we summarize the current recommendations about the use of metformin in CKD.

All diabetic subjects at risk of acute renal failure must discontinue at least temporally metformin. Clinical situations related to increase of acute renal failure includes hepatic insufficiency and use of radiocontrast agents and antimicrobial drugs. Fluid substitution as well as support of cardiac output is useful in certain clinical conditions. Monitoring of urine output and serum creatinine lack sensitivity and specificity in acute renal failure, they remain the most used parameters in clinical practice.

At last, when we change the dose of drugs affecting blood pressure and potentially renal perfusion we have to monitor renal function closely and to reduce the dose of metformin (use of diuretics or increase of their dose,

Table 1 Use of metformin in chronic kidney disease

eGFR (mL/min per 1.73 m^2)	Use of metformin
> 60 (CKD 1 and 2)	No contraindication Check of renal function annually
45-60 (CKD 3a)	Use of metformin-reduce dose (no more than 1.5-2 g daily) Frequent check of renal function (every 3-6 mo)
30-45 (CKD 3b)	Reduce dose (no more than 1-1.5 g daily) No new cases Frequent check of renal function (every 3-6 mo)
< 30 (CKD 4 and 5)	Stop metformin

CKD: Chronic kidney disease; eGFR: Estimate Glomerular Filtration Rate.

start of use of ACEIs and ARBs, unstable heart failure with frequent hospitalizations, *etc.*).

PIOGLITAZONE

Pioglitazone has only and exclusively hepatic metabolism. It does not cause hypoglycemia and it can be given theoretically without dose adjustment at all stages of CKD. Pioglitazone is related with fluid retention, anemia and osteoporosis. These side effects complicate the existing problems with anemia and bone disease in subjects with diabetes and CKD[15,16].

The use of pioglitazone is generally limited in these patients and in decreased dose (usually 15 mg once daily).

SULFONYLUREAS

Sulfonylureas are old drugs widely used worldwide. These drugs ease the secretion of insulin and are related with increased risk of hypoglycemia, which is a major issue for CKD patients.

Glibenclamide

Glibenclamide (glyburide) is metabolized in the liver and excreted by the kidneys equally and intestine. Some metabolites are active and can accumulate in CKD despite the fact that biliary removal partially counteracts the limited renal excretion.

Hypoglycemia may be serious and lasting more than 24 h in CKD.

The use of glibenclamide in subjects with moderate CKD (eGFR 60-90 mL/min) should be limited (reduced dose, frequent monitoring due to increased risk of hypoglycemia). The drug and is contraindicated in stage $\geqslant$ 3 CKD (eGFR < 60 mL/min)[17].

Glimepiride

Glimepiride is metabolized by the liver to two major metabolites each of which has hypoglycemic activity. In renal disease these metabolites summed. Although the half-life is 5-7 h, the drug can cause severe hypoglycemia that lasts more than 24 h. Its use is safe in GFR > 60 mL/min and with a reduced dose of up to 30 mL/min. In CKD stage 4 or 5 the use of glimepiride is dangerous[18].

Gliclazide

Gliclazide is metabolized by the liver to inactive metabolites that are eliminated in the urine. Thus, gliclazide causes less hypoglycemia than other sulfonylureas. In CKD sage 1, 2, 3 (eGFR > 30 mL/min) gliclazide can be used. There are no data in patients with severe CKD but according to its metabolism the use (in reduced dose) of gliclazide is also permitted in these subjects[19].

Glipizide

Glipizide also does not need dose adjustment in severe and moderate renal disease and can be used safely. (The only caution remains the risk of hypoglycemia).

GLINIDES

Glinides, repaglinide and nateglinide, are short acting secretagogues. The short duration of their action means reduced risk of hypoglycemia compared to sulfonylureas. This is an advantage for diabetic subjects with CKD because they belong in the high risk for hypoglycemia group as already mentioned.

Repaglinide is absorbed from the gastrointestinal tract and metabolized in the liver by oxidation and conjugation with glucuronic acid. The major metabolites of repaglinide are M1, M2 and M4. These metabolites are excreted *via* the bile into the feces and have no hypoglycemic activity[20].

Repaglinide can be used even in CKD stages 4 and 5 without dose reduction.

Nateglinide is also rapidly absorbed from the gastrointestinal tract and metabolized in liver to 9 main metabolites (M1-M9). These metabolites have much weaker hypoglycemic activity than the parent compound. The only metabolite that retains high activity is the metabolite M7. The concentration of this metabolite however is low (< 7%), resulting in a hypoglycemic effect, which is attributed mainly to intact nateglinide. The excretion of the drug in urine is unchanged form at 16% and by 84% in the form of metabolites.

In CKD stage 5 we avoid nateglinide, and in stage 4 we adjust the dose (60 mg × 3)[21].

GLIPTINES (DIPEPTIDYL PEPTIDASE 4 INHIBITORS)

Dipeptidyl peptidase 4 (DPP-4) inhibitors (gliptins) constitute a new class of antidiabetic drugs with a very favorable profile: safety, efficacy, and low risk of hypoglycemia and weight neutrality[22].

Gliptins are inhibitors of the enzyme DPP-4. This enzyme degrades and inactivates many active peptides. Among them are incretin hormones. These hormones, namely glucagon like peptide 1 (GLP-1) and glucose dependent insulinotropic polypeptide stimulates glucose

Table 2 Dose adjustment of dipeptidyl peptidase 4 inhibitors in chronic kidney disease

	CKD			
	CKD 1, 2 and 3a (Cl_{cr} > 50 mL/min)	CKD 3b (Cl_{cr} 30-50 mL/min)	CKD stage 4 (Cl_{cr} 15-30 mL/min)	CKD stage 5 (ESRD)
Sitagliptin (Januvia)	√ (100 mg × 1)	1/2 dose (50 mg × 1)	1/4 dose (25 mg × 1)	1/4 dose (25 mg × 1)
Vildagliptin (Galvus)	√ (50 mg × 2)	50 mg × 1		50 mg (no experience)
Saxagliptin (Onglyza)	√ (5 mg × 1)	1/2 dose (2.5 mg × 1)	1/2 dose (2.5 mg × 1)	1/2 dose (2.5 mg × 1)
Linagliptin (Trajenta)	√ (5 mg × 1)	√ (5 mg × 1)	√ (5 mg × 1)	P (5 mg × 1)
Alogliptin (Nesina)	√ (25 mg × 1)	1/2 dose (12.5 mg × 1)	1/4 dose (6.25 mg × 1)	1/4 dose (6.25 mg × 1)

CKD: Chronic kidney disease; ESRD: End stage renal disease.

dependent insulin secretion by b cells in pancreatic islets. At the same time they suppress glucagon production by a cells in the same islets. Their role in glucose homeostasis seems to be important. These hormones are secreted in low levels when we are fasting but their secretion is rapidly increased after meal consumption. Their action results also in reduced glucagon secretion, which in turns reduces hepatic glucose production.

Vildagliptin, Sitagliptin, Saxagliptin, Linagliptin and Alogliptin belong to this class and are already available in the market. Their place in algorithms for patients with diabetes and CKD is important. We can use them all in CKD but with dose adjustment for the majority of the members of this class. (Only linagliptin does not need dose adjustment in any stage of CKD)[23].

In Table 2 we summarize the dose adjustments for all gliptins in diabetic subjects with CKD.

Sitagliptin

Sitagliptin does not undergo extensive metabolism. In the liver sitagliptin partially metabolized by oxidation in a limited rate by the enzyme CYP3A4. Nevertheless, most of the drug is excreted in the intact form in the urine (more than 80%). Sitagliptin is filtered in renal glomerulus but also is actively excreted by active tubular secretion[24].

Six metabolites are detected in amounts of < 1% to 7%. These metabolites M1 to M6 are products of hepatic metabolism.

Chemically the changes in these metabolites are: M1: N-sulfation, M4: N-carbamyl glucuronidation, M6: hydroxylation followed by either glucuronidation (M3), and oxidative desaturation followed by cyclization (M2 and M5). These metabolites are practically inactive.

In renal disease the elimination of the drug is reduced resulting in 2- or 4-fold increase of the concentration of the drug (for Cl_{cr} 30-50 mL/min and < 30 mL/min respectively). The dose adjustment is based on these properties.

In Phase I studies of sitagliptin dosing up to 600 mg daily doe not results to dose-related side effects, at least in the short term (up to 28 d). These data indicates that if we don't adjust the dose in CKD practically it might be safe at least for a short period[25,26].

Vildagliptin

Vildagliptin is absorbed quickly (85.4% of the drug). The maximum plasma level is detected at 1.1-h post dose.

Plasma radioactivity (after the administration of radioactive labeled drug) due to vildagliptin is 25.7% and to its major metabolite M20.7 is 55%. The half- life of vildagliptin is 2.8 h. Eighty-five percent of the drug is excreted in the urine (22.6% as vildagliptin the rest as inactive metabolites) and the remaining 15% in feces (4.54% as vildagliptin). In humans, the main pathway of metabolism of the drug is carboxylation, which results in the form of the active metabolite M20.7. DPP-4 contributes to formation of this metabolite. Other minor metabolites are: M15.3, which results from hydrolysis of amide bonds, M20.2 from glucuronidation of the pyrrolidine ring and M20.9, M21.6 from oxidation of the pyrrolidine ring. All these metabolites are inactive[27].

Hydrolysis takes place in multiple tissues or organs. Exposure to vildagliptin in subjects with type 2 diabetes and renal disease of various stages cannot be accurately predicted because the kidneys play a small role in the removal of the drug while participating in metabolism *via* hydrolysis[28].

In diabetic subjects with chronic kidney disease stage 1 or 2 (eGFR > 50 mL/min per 1.73 m^2), dose adjustment of vildagliptin is not required.

In patients with chronic kidney disease stage ⩾ 3, both vildagliptin and its active metabolite M20.7 are less excreted *via* the kidneys. In these patients a dose adjustment is required. (When eGFR is < 50 mg/mL per 1.73 m^2 the dose is 50 mg × 1).

Saxagliptin

Saxagliptin is primarily hepatic metabolized by the cytochrome P450 3A4/5 (CYP3A4/5). The major metabolite of this drug is also active as also a DPP-4 inhibitor, and retains half of the potency of parent drug.

All the drugs, which are also metabolized in this cytochrome CYP3A4/5, may alter the pharmacokinetics of the drug and its active metabolite. Twenty-four percent of the drug is excreted in the urine as saxagliptin and 36% as its active metabolite. There is also some active renal excretion of the drug. A significant part (more than 20%) can be found in the feces as a sum of excreted in bile drug and unabsorbed drug[29].

In diabetic patients with chronic kidney disease stages 1 and 2 increased concentration of saxagliptin and its active metabolite remains clinically irrelevant and no dose adjustment is needed.

In diabetic subjects with chronic kidney disease stages ⩾ 3 half dose is recommended (2.5 mg × 1 daily) to

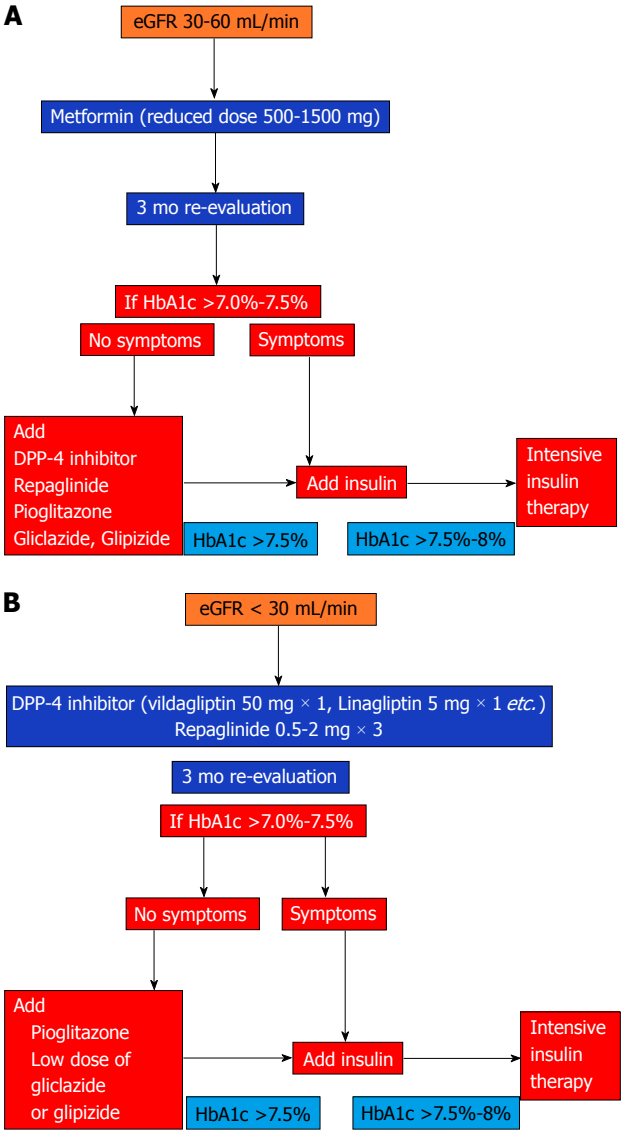

Figure 1 Therapeutic algorithm (A and B). eGFR: Estimate Glomerular Filtration Rate; HbA1c: Haemoglobin A1c; DPP-4: Dipeptidyl peptidase 4.

achieve the same plasma concentrations compared to subjects with normal renal function. The same dose is recommended in patients with end-stage renal disease (requiring hemodialysis).

Alogliptin

This DPP-4 inhibitor is not practically metabolized and is excreted unchanged in the urine. (More than 70% of the parent drug). One minor metabolite named M1 is active but its concentration remains quite low (< 1%)[30]. Alogliptin is excreted by glomerulus filtration as well as by active tubular secretion.

In patients with CKD stage 1 and 2 no dose adjustment is needed (25 mg × 1 daily). In patients with CKD stage 3 (Cr_{Cl} ⩾ 30 to < 60 mL/min), the recommended dose is 12.5 mg once daily and in patients with CKD stage ⩾ 4 the recommended dose is 6.25 mg once daily. The same dose is required in patients with end-stage renal disease requiring dialysis.

Linagliptin

Linagliptin is primarily nonrenally excreted: 80% of the drug is eliminated *via* the bile and gut and only 5% is eliminated *via* the kidney[31]. The drug is not practically metabolized and is excreted unchanged. There is no need of dose adjustment in any stage of CKD (5 mg × 1 for all diabetic subjects).

GLP-1 RA (RECEPTORS AGONISTS)

These drugs are injectable and are potent without risk of hypoglycemia. They have to be used with caution in patients with CKD because their gastrointestinal side effects can induce deterioration of renal disease. (Dehydration due to vomiting or diarrhea).

Exenatide

Exenatide is excreted only by the kidneys and undergoes fragmentation in the renal tubule. It does not metabolized by DPP-4 nor the neutral endopeptidase (NEP). There is no hepatic metabolism of exenatide[32].

In CKD stage 3 dose reduction is needed (5 μg × 2 and close monitoring). In CKD stage 4 and 5 (clearance < 30 mL/min) is not allowed.

Liraglutide

Liraglutide is cleaved *in vivo* by the enzyme DPP-4 that elicits two amino acids at the N terminus of the peptide. NEP also metabolizes liraglutide into several metabolites[33].

Of the administered drug (radioactive labeled) only 26.3% appears in the urine and feces, while breathing excretes 15%. Twenty point one percent of radioactivity is excreted in the urine mainly as water and only 6.3% in substances other than water.

Liraglutide is degraded entirely in the body and is not excreted in urine and feces. These characteristics indicate that we can use in all stages of CKD. Nevertheless we have not yet clinical studies in patients with eGFR < 60 mL/min[34] (there is ongoing studies with preliminary, not yet published, positive results of safety and effectiveness in patients with CKD stage ⩾ 3).

INSULIN

The kidneys carry out one third of exogenous insulin degradation. It is filtered at the glomerulus and is absorbed by the proximal tubule. Sixty percent of the renal clearance is due to glomerular filtration and 40% in the secretion by uptake from peritubular vessels. Reduction in renal filtration is partially counterbalanced by secretion[35]. The dose of exogenous insulin is reduced 25% when eGFR is 10-50 mL/min and 50% when eGFR is < 10 mL/min[36].

CONCLUSION

The landscape is not clear enough in diabetes treatment in CKD. The risk of hypoglycaemia, which is higher in

subjects with both diabetes and CKD, leads to selection of appropriate drugs with low risk of hypoglycaemia such as metformin (reduced dose) and DPP-4 inhibitors. When insulin treatment is appropriate, dose adjustment is usually required especially in CKD stages 4 and 5. Finally, many people with diabetes have a less strict target of glycaemia.

ACKNOWLEDGMENTS

Based on all these data I propose the algorithms as shown in Figure 1.

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P- Reviewer: Mitra A, Tamemoto H, Zhang Q
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Biomarkers in diabetic nephropathy: Present and future

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Received: August 25, 2014 Revised: October 3, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease in the Western world. Microalbuminuria (MA) is the earliest and most commonly used clinical index of DN and is independently associated with cardiovascular risk in diabetic patients. Although MA remains an essential tool for risk stratification and monitoring disease progression in DN, a number of factors have called into question its predictive power. Originally thought to be predictive of future overt DN in 80% of patients, we now know that only around 30% of microalbuminuric patients progress to overt nephropathy after 10 years of follow up. In addition, advanced structural alterations in the glomerular basement membrane may already have occurred by the time MA is clinically detectable.Evidence in recent years suggests that a significant proportion of patients with MA can revert to normoalbuminuria and the concept of nonalbuminuric DN is well-documented, reflecting the fact that patients with diabetes can demonstrate a reduction in glomerular filtration rate without progressing from normo-to MA. There is an unmet clinical need to identify biomarkers with potential for earlier diagnosis and risk stratification in DN and recent developments in this field will be the focus of this review article.

Key words: Diabetes; Nephropathy; Microalbuminuria; Proteinuria; Biomarkers

Core tip: Microalbuminuria (MA) is the earliest and most commonly used clinical index of diabetic nephropathy (DN), however its sensitivity and specificity for early disease detection are limited. Not all patients with MA progress to overt DN, nonalbuminuric DN is common and risk associated with MA is elevated even at levels below currently accepted diagnostic thresholds. There is therefore a need for alternative biomarkers allowing early identification of "at risk" individuals. This review focusses on biomarkers of glomerular and tubular dysfunction, oxidative stress and inflammation that have attracted interest. In addition we review more novel strategies including proteomic, metabolomic and genomic approaches.

Original sources: Currie G, McKay G, Delles C. Biomarkers in diabetic nephropathy: Present and future. *World J Diabetes* 2014; 5(6): 763-776 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/763.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.763

INTRODUCTION

The global incidence of type 2 diabetes continues to rise due to the increase in obesity and the aging population. In 2000 the prevalence of diabetes was estimated to be 171 million (2.8%) worldwide. It is projected that by 2030, 366 million (4.4%) people worldwide will have diabetes[1,2]. Diabetic nephropathy (DN), defined as albuminuria (albumin excretion rate > 300 mg/24 h) and declining renal function in a patient with known diabetes in the absence of urinary tract infection or any other renal

disease[3], is the leading cause of end stage renal disease in the Western world. In the 1960s the development of assays for detection of microalbuminuria (MA) revolutionised diabetes management[4]. MA, defined as urinary albumin excretion rate (UAE) 30-300 mg/d, is the earliest and most commonly used clinical index of DN. MA is independently associated with cardiovascular risk in diabetic patients[5-8], due in part to its role as an indicator of widespread microvascular disease and of underlying renal disease, and studies have since indicated that a reduction of UAE in type 2 diabetic patients reflects renal and cardiovascular risk reduction[9]. Consequently, UAE has become a key therapeutic target in the management of patients with diabetes. Evidence from the Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study Group proved that tight glycaemic and blood pressure control can reduce risk of microvascular complications of diabetes including DN[10-12] for patients with type 1 or type 2 diabetes respectively and this strategy forms the basis of current management guidelines for microalbuminuric patients.

Although UAE remains an essential tool for risk stratification and monitoring disease progression a number of factors have called into question its sensitivity and specificity. The presence of MA was originally thought to be predictive of future overt DN in 80% of patients. However more recent evidence suggests that only around 30% of microalbuminuric patients progress to overt nephropathy after 10 years of follow up[13]. It has also been shown that advanced structural alterations in the glomerular basement membrane may already have occurred by the time MA becomes clinically evident[14,15]. In addition, there is evidence that a significant proportion of patients with MA can revert to normoalbuminuria[16] and the concept of nonalbuminuric DN is well-documented, reflecting the fact that patients with diabetes can demonstrate a reduction in glomerular filtration rate without progressing from normo-to MA[14,17]. Taken together, these results suggest that MA is perhaps more a diagnostic marker than a tool to predict DN. Therefore, there is a need to identify and investigate alternative biomarkers for the earlier prediction of DN and these are subject to this review.

GLOMERULAR FILTRATION

Glomerular filtration rate (GFR) is the best marker of renal excretory function. The current gold standard methods for determining GFR in the research setting are inulin and ^{51}Cr-EDTA plasma clearance. The time-consuming and labour intensive nature of these techniques, as well as requirement of experienced personnel, however, mean that they are not routinely available in clinical practice. Here the most commonly used index for assessment of GFR is serum creatinine, although its sensitivity is poor in the early stages of renal impairment, as by the time an increase in serum level is detectable, a significant decline in GFR has already taken place[18]. Formulae using serum creatinine to estimate GFR (eGFR) such as the Modification of Diet in Renal Disease equation are not reliable at GRF > 60 mL/min per 1.73 m^2. The recently developed Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula appears to be more accurate in patients whose GFR is > 90 mL/min per 1.73 m^2[19-21] however a marked underestimation of GFR in diabetic patients continues to be evident using this equation when compared to its performance in healthy individuals[22]. The current Kidney Disease Improving Global Outcomes guidelines staging system classifies chronic kidney disease stages 1 and 2 using GFR cut-offs of > 90 mL/min and 60-89 mL/min respectively[23]. Routine clinical tests therefore do not measure this degree of GFR decline accurately, meaning that this potentially critical early stage of renal dysfunction remains undetected[24].

Cystatin C (CysC) based assays in estimating GFR for clinical trials in DN offer an alternative approach due to the complexity and time-consuming nature of other reference test methods. This 13.3 kDa plasma protein is freely filtered through the glomerulus and reabsorbed and catabolised by tubular cells to such a degree that it does not return to the blood in an intact form[25]. Numerous studies have validated CysC as a marker of renal function[26-28]. Its levels are well correlated with GFR and unlike serum creatinine, are unaffected by muscle mass. In addition CysC levels not only correlate with progression of nephropathy, but also show a more sensitive marker of early DN when eGFR remains > 60mL/min[29-31]. These benefits should, however, be taken into consideration alongside the higher cost of the immunoassay and the greater intraindividual variability[28] compared to serum creatinine Formulae for estimating GFR including both creatinine and CysC have been proposed but to date have not been proven to enhance precision in identifying and monitoring early stages of GFR decline in diabetes[32].

MARKERS OF GLOMERULAR DYSFUNCTION

Glomerular damage increases permeability to plasma proteins resulting in their excretion in the urine. In addition, abnormalities of extracellular matrix synthesis and degradation in kidney disease can lead to increased urinary excretion of matrix proteins, reflecting glomerular injury. Although albumin excretion remains the current gold standard marker of glomerular damage in the clinical setting, a number of other proteins have been proposed as useful indicators of early glomerular damage.

Transferrin is a plasma protein with a slightly greater molecular weight (76.5 kDa) than albumin[33]. It is also less ionic than glycosylated albumin and thus less easily repelled by glomerular basement membrane polyanion[34]. Elevated urinary transferrin excretion has been demonstrated in patients with diabetes compared with healthy controls, even in in the absence of albuminuria[35]. Transferrinuria has been shown to correlate with UAE and to increase in parallel with it[36]. In a 24 mo follow up study it

has been demonstrated that increased urinary transferrin excretion predicted development of MA in a cohort of normoalbuminuric type 2 diabetic patients independent of age, diabetes duration, blood pressure, HbA1c and baseline lipid levels[33]. Elsewhere it has also been shown that transferrinuria predicted development of MA at 5 years follow up[36]. Transferrin has also been proposed as a mediator of tubular toxicity, as its reabsorption results in release of reactive iron in proximal tubular cells promoting formation of hydroxyl radicals[37,38]. Studies have reported correlations between urinary transferrin excretion and other microvascular diabetic complications such as retinopathy[38]. Taken together, the above data suggest that transferrinuria may serve as a sensitive indicator of early proteinuria and increased vascular permeability.

Accumulation and altered distribution of basement membrane components is one of the structural hallmarks of DN and these changes precede the development of MA[39]. Type Ⅳ collagen is a normal constituent of mesangial matrix as well as tubular and glomerular basement membranes, with molecular weight of 540 kDa. Both serum and urine levels have been shown to be elevated in patients with diabetes[40]. Urinary type Ⅳ collagen excretion has been shown to correlate closely with degree of UAE, as well as diabetes duration, blood pressure and serum creatinine[41,42]. Significantly higher excretion of type Ⅳ collagen has been found even in normoalbuminuric diabetic patients as well as patients with impaired glucose tolerance, suggesting that this may serve as an early indicator of DN, preceding the onset of MA[42,43]. In addition, type Ⅳ collagen excretion has been found to decrease with improved glycaemic control, suggesting that this marker is also reversible in early disease[44]. Type Ⅳ collagen may also play a role in differentiating DN from other non-diabetic kidney diseases, as the ratio of type IV collagen to albumin has been found to be significantly higher in DN in comparison to other glomerulopathies[40].

Ceruloplasmin is a 132 kDa acute phase protein with well characterised functions in the metabolism of copper and iron[36]. It has been suggested that ceruloplasmin may leak through glomerular capillary walls in DN and evidence confirms increased excretion in both impaired glucose tolerance and diabetes compared with healthy controls[36,45]. Increased urinary ceruloplasmin excretion has also been demonstrated in normoalbuminuric patients with diabetes[45]. In addition, urinary ceruloplasmin excretion appears to parallel UAE[31,46]. In a 5 year follow up study, it was demonstrated that increased urinary ceruloplasmin excretion predicted development of MA in normoalbuminuric type 2 diabetic patients[36]. Improved glycaemic control appears to reverse this increase[46].

Fibronectin is a high molecular weight (440 kDa) plasma glycoprotein mainly produced by endothelial cells and fibroblasts which plays a role in cell adhesion to vascular endothelium[35]. Fibronectin biosynthesis is increased in patients with diabetes and studies have suggested that plasma levels correlate with retinopathy and MA[47]. Increased urinary levels of fibronectin have been found in type 2 diabetic patients in comparison with healthy controls, as well as in subjects with MA compared to normoalbuminuric subjects[47]. However, there is only a weak positive correlation between plasma fibronectin and urinary albumin levels perhaps limiting its potential usefulness as an early marker of DN[47], and there is no published evidence comparing urinary fibronectin with UAE in terms of predictive value for diabetic nephropathy.

MARKERS OF TUBULAR DYSFUNCTION

Plasma proteins of low molecular weight are excreted in increased quantities in the urine due to deficient tubular reabsorption or increased secretion by tubular epithelial cells. Similarly, urinary enzymes are thought to be sensitive markers of tubular damage as they are not filtered at the glomerulus due to their high molecular weight[31,36].

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a small molecule of 25 kDa belonging to the lipocalin superfamily. These proteins play a role in binding and transporting small hydrophobic molecules, apoptosis and immune regulation. NGAL is stored mainly in the specific granules of neutrophils and also expressed at low levels in several other human tissues[48,49]. NGAL shows significant promise in the diagnostic and clinical setting as a marker of acute kidney injury[48] and is thought to also play a renoprotective role as a mediator of tubular cell proliferation[49]. Studies have confirmed an association between NGAL and obesity, insulin resistance and hyperglycaemia in human subjects[49]. Urinary NGAL concentration has been found to be increased in diabetic subjects compared with healthy controls[50] and to correlate negatively with eGFR, and positively with CysC, serum creatinine and urea in patients with type 2 diabetes[48]. Significant increases in urinary NGAL concentration have been demonstrated from normo- to micro- to macroalbuminuric groups of patients with type 1 diabetes[51]. Similar results have been published in a study of type 2 diabetic patients[52]. Urinary NGAL correlates positively with glomerular hyperfiltration early in the clinical course of diabetes[53] and higher values have been found to be associated with enhanced decline in eGFR in type 2 diabetes patients with proteinuria, although this correlation was no longer statistically significant after adjustment for factors including systolic blood pressure, HbA1c and diabetes duration[53]. However, other prospective studies have not confirmed these associations[54,55] and further investigation of the role of urinary NGAL in DN is required.

Kidney injury molecule 1 (KIM1) has been shown to be a marker of tubular damage in various chronic kidney diseases[56,57]. This type 1 cell membrane glycoprotein is expressed on the apical membrane of proximal tubule cells and is involved in the phagocytosis of damaged cells in the proximal tubules[52]. Expression is undetectable in normal healthy kidneys but mRNA and protein are markedly upregulated in acute kidney injury[58]. In a cross-sectional study urinary KIM1 excretion has been

found to be increased in diabetic patients compared to healthy controls. A weak but significant increase of urinary KIM1 concentration was noted with increasing degree of UAE[50]. Increased urinary KIM1 excretion has also been shown in type 2 diabetics with glomerular hyperfiltration[52]. In a 3 year prospective interventional study, high baseline levels of urinary KIM1 were found to be associated with faster decline in GFR in type 1 diabetes with DN; an association no longer significant after adjustment for traditional risk markers including blood pressure and glycaemic control[58]. Similar findings have been described in type 2 diabetes populations[55]. Studies have shown that treatment with renin angiotensin system (RAAS) blocking agents reduced urinary KIM1 excretion in parallel to reductions in blood pressure and UAE[59]. In addition, low baseline urinary KIM1 excretion is strongly associated with regression of MA during a 2 year follow up period, independent of clinical characteristics[57]. This supports the hypothesis that KIM1 is a good marker of active tubular damage, rather than pre-existing scarring[58].

N-acetyl-b-d-glucosaminidase (NAG) is a lysosomal enzyme which is predominantly located in the renal tubules. It cannot be filtered from blood through an intact glomerular membrane due to its high molecular weight (140 kDa), thus its activity detected in urine reflects tubular dysfunction. Urinary NAG activity is increased in a variety of tubulointerstitial diseases. It is elevated in populations with diabetes compared to controls, even in normoalbuminuric patients[33,53]. It correlates with the degree of UAE and excretion of transferrin and creatinine[60-62]. Although no significant association has been found between urinary NAG and glomerular hyperfiltration[52], prospective follow up studies have shown that higher levels of NAG at baseline are predictive of subsequent DN[63]. In addition, lower baseline NAG levels are significantly associated with regression of MA at follow up[57]. Finally, significant increases in NAG excretion have been reported in type 2 diabetic patients with both micro- and macrovascular complications[63-65] and in fact NAG levels have been attributed comparable diagnostic value to UAE in this regard[65].

Liver-type fatty acid binding protein (L-FABP) is a low molecular weight (15 kDa) intracellular carrier protein that is expressed in the proximal tubule and liver[66,67]. It is produced in response to tubulointerstitial compromise, and thus has potential as a marker of structural and functional renal tubular damage[67]. In a cross sectional study of patients with type 1 diabetes and varying degrees of UAE, urinary L-FABP levels were significantly higher compared to healthy controls. The levels increased with increasing degree of albumin excretion. Intervention with Lisinopril was associated with significant reductions in UAE and urinary L-FABP excretion in those with diabetes[68]. However, there is no correlation between L-FABP and rate of change of eGFR in patients with type 2 diabetes[54]; therefore further studies are needed to elicudate its value as a predictive marker for DN.

Low molecular weight proteins are freely filtered at the glomerulus and some have been used as markers of tubular damage in various renal diseases[36]. β2-microglobulin (β2MG) is a 11.8 kDa protein produced by cells expressing major histocompatibility class 1. Urinary β2MG excretion is known to be elevated in patients with reduced GFR and some evidence links β2MG with tubular injury[69]. β2MG has also been associated with macrovascular complications in type 2 diabetes[63]. However, its diagnostic utility is limited by its poor stability at acidic pH[70]. The stable microprotein α-1-microglobulin (A1M) may offer an alternative means of evaluating tubular function. This 26 kDa glycoprotein is freely filtered at the glomerulus and almost completely reabsorbed in the proximal tubules, thus even minor degrees of proximal tubular dysfunction lead to increased urinary A1M excretion[71,72]. Urinary A1M excretion has been shown to be greater in patients with type 2 diabetes compared to healthy controls[33,42]. A1M levels have also been found to correlate with diabetes duration and degree of diabetes control[63,71]. There is evidence that urinary A1M excretion significantly increases with degree of MA in type 2 diabetes[71-73]. However, Hong *et al*[72] found in a cross-sectional study that although UAE and A1M were directly related, in some patients one could be present in the absence of the other, suggesting that urinary A1M (as a measure of tubular function) may be complementary to MA (as a measure of glomerular function) in assessment of early DN. Retinol binding protein (RBP) is another low molecular weight protein (21 kDa) which is freely filtered at the glomerulus and almost completely reabsorbed in the proximal tubule; as such its presence in the urine is indicative of even very minor degrees of tubular dysfunction[33]. Increased urinary RBP excretion has been described in diabetic patients compared to controls, even in patients with normal UAE[16,70,73]. RBP levels have also been found to correlate with both micro- and macrovascular complications in type 2 diabetic patients[64,74]. RBP, therefore, may also have a complementary role in early detection of DN together with biomarkers of glomerular damage such as UAE or transferrin. Immunoglobulin free light chains (FLCs) kappa and lambda undergo similar glomerular filtration and near complete tubular reabsorption[36]; consequently their presence in the urine can also be indicative of proximal tubular dysfunction[75]. Abnormal urinary FLCs/creatinine ratio in type 2 diabetes patients, both with normal and elevated UAE, and FLC excretion appears to be increased before overt renal disease occurs[76]. However, as yet there is little further published evidence regarding use of FLCs as a predictive tool for early detection of DN.

MARKERS OF OXIDATIVE STRESS AND INFLAMMATION

Oxidative stress is thought to be one of the key mediators of vascular complications of diabetes. Generation of reactive oxygen species (ROS) as a result of hyperglycaemia contributes to development of diabetes com-

plications through sorbitol accumulation, formation of advanced glycation end products (AGE) and activation of protein kinase C[77,78].

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage resulting from specific enzymatic cleavage after ROS-induced 8-hydroxylation of the guanine base in nuclear and mitochondrial DNA[78]. Since it is excreted into urine without being further metabolised its urinary concentration serves as an index of oxidative stress[79]. Increased concentrations of 8-OHdG have been described in both urine and mononuclear cells of diabetic patients[80], and urinary excretion appears to correlate closely with the severity of DN and retinopathy as well as HbA1c[81]. In a prospective longitudinal study of 532 Japanese diabetic patients, urinary 8-OHdG excretion at baseline was associated with later development of DN after 5 years of follow up[81], indicating its potential as a clinical predictive marker.

AGE have been associated with the pathogenesis of diabetes complications[82]. AGE-modified proteins generally undergo glomerular filtration and subsequent catabolism at the proximal tubule, thus it seems intuitive that the presence of AGE-modified protein fragments in urine may also herald early tubular dysfunction**.** Pentosidine is one of the major molecular structural components of AGEs and acts as a marker of their formation and accumulation[83]. Urinary excretion of Pentosidine has been shown to be higher in patients with diabetes compared to healthy controls[84]. Increased urinary and plasma Pentosidine levels have been demonstrated in patients with DN[85]. More recently its potential as a marker of microvascular complications of diabetes has been shown with associations between serum Pentosidine levels and diabetic retinopathy, hypertension and hyperlipidaemia in addition to DN[86]. Although initially no correlation between Pentosidine levels and UAE were reported[84], recent publications have challenged this finding; one study reported significantly increased serum Pentosidine levels in diabetes patients with MA compared to normoalbuminuric controls[87] and another study found increased median urinary Pentosidine excretion in diabetes patients with macroalbuminuria compared to controls[62]. In addition, this study demonstrated that baseline urinary Pentosidine excretion predicted later macroalbuminuria, with risk increasing almost 7-fold for every 50% increase in urinary Pentosidine[62].

Evidence is accumulating that immune and inflammatory mechanisms also play a role in the pathogenesis of DN[88], as cause rather than consequence of disease[89]. Individuals who progress to DN appear to display features of low grade inflammation for years before clinically detectable disease[90,91]. As a result, cytokines and other components involved in the process of inflammation and endothelial damage have attracted attention as potential markers of DN.

Orosomucoid, or α-1-acid glycoprotein (AGA) is a single chain polypeptide produced mainly by the liver. It is released in response to inflammation under the stimulation of cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α)[92]. AGA levels have been found to be associated with ischaemic heart disease, lung cancers and diabetes[92,93]. It has been suggested that high AGA levels may predict the development of type 2 diabetes[94]. In a cross sectional study of outpatients with type 2 diabetes and no known cardiovascular disease, serum AGA levels were found to correlate significantly with UAE[95]. In addition, proteomic work has identified urinary AGA as an independent risk factor for DN[96,97]. Urinary AGA excretion appears to increase in parallel with UAE and data indicate that urinary AGA is elevated in the early stages of DN[95]. The potential predictive value of urinary AGA in DN has been shown[98] but further work is needed to determine whether AGA could be used as a biomarker of disease development and treatment response.

TNF-α and IL-6 are two major pro-inflammatory cytokines that stimulate the acute phase response by triggering production of other proteins such as CRP and AGA[89,93]. Patients with DN have higher serum and urinary concentrations of TNF-α than healthy controls or normoalbuminuric subjects[99,100]. Urinary TNF-α excretion also appears to be increased in diabetes patients with micro- or macroalbuminuria compared to normoalbuminuric patients[100,101], with one study reporting an increase of 90% between normo- and microalbuminuric patients[100]. Urinary TNF-α excretion has also been shown to correlate with NAG excretion, a marker of severity of tubular damage[99]. TNF-α mediates its effects *via* two distinct receptors, TNF receptor 1 (TNFR1) and TNFR2, which are both membrane bound and also can be found in serum in soluble form[102]. Serum levels of both these receptors have been shown to correlate with GFR in diabetic patients independently of albuminuria status[102]. More recent data suggest that serum concentrations of TNFR1 and TNFR2 have potential as predictors of progressive renal disease in diabetes[103,104]. Patients with TNFR levels in the highest quartile show significantly elevated cumulative incidence of reaching stage 3-5 CKD over 12 years of follow up compared with those in the lower quartiles. This has been shown in both type 1 and type 2 diabetes, in the presence or absence of proteinuria[103,104].

Serum IL-6 has been shown to be elevated in patients with diabetes compared to control subjects, as well as between normo-, macroalbuminuric and overtly proteinuric patient groups[105,106]. In addition, IL-6 has been linked to glomerular basement membrane thickening[106]. Furthermore, association has been demonstrated between circulating levels of both TNF-α and IL-6 and micro- and macrovascular complications of diabetes[107].

Vascular endothelial growth factor (VEGF) is a potent cytokine that induces angiogenesis and increases endothelial permeability[108]. It adversely affects the glomerular filtration barrier by enhancing its permeability to macromolecules and exacerbating proteinuria[109]. Urinary VEGF excretion appears to be elevated in patients with

Table 1 Summary of biomarkers with potential utility in diagnosis of diabetic nephropathy

Biomarker	Serum/plasma or urine	Type of marker	Status in DN	Potential for additional information beyond UAE	Ref.
Transferrin	Urine	Glomerular	Elevated	Predicts MA	[30-35]
Type Ⅳ collagen	Urine	Glomerular	Elevated	Rises in parallel with UAE, even in nonalbuminuric stage	[36-41]
Ceruloplasmin	Urine	Glomerular	Elevated	Predicts MA	[33,42-44]
Fibronectin	Plasma/urine	Glomerular	Both elevated	No	[32,45]
NGAL	Urine	Tubular	Elevated	Marker of glomerular hyperfiltration	[46-53]
KIM1	Urine	Tubular	Elevated	Marker of glomerular hyperfiltration	[49,50,53-57]
NAG	Urine	Tubular	Elevated	Comparable to UAE	[30,58-64]
L-FABP	Urine	Tubular	Elevated	No	[52,65-66]
A1M	Urine	Tubular	Elevated	No	[30,39,63,69-74]
RBP	Urine	Tubular	Elevated	No	[17,30,69,72-75]
FLCs	Urine	Tubular	Elevated	No	[17,63,69,72-75]
8-OHdG	Urine	Oxidative stress	Elevated	Predicts DN but value in comparison to MA remains unclear	[77-80]
Pentosidine	Urine/serum	Oxidative stress	Both elevated	No	[61,81-86]
AGA	Urine	Oxidative stress	Elevated	Urinary excretion predicts MA	[91-97]
TNF-α	Urine/serum	Inflammatory	Both elevated	No	[88,92,98-100]
TNFR 1/2	Serum	Inflammatory	Elevated	Predictive of onset of stage 3-5 CKD independent of albuminuria status	[99-101]
IL-6	Urine/serum	Inflammatory	Serum levels elevated	No	[99,101-103]
VEGF	Urine/serum	Inflammatory	Urinary levels elevated	No	[104-108]

DN: Diabetic nephropathy; NGAL: Neutrophil gelatinase associated lipocalin; KIM1: Kidney injury molecule 1; NAG: N-acetyl-b-d-glucosaminidase; AIM: α-1-microglobulin; L-FABP: Liver type fatty acid binding protein; RBP: Retinol binding protein; FLCs: Free light chains; 8-OHdG: 8-oxo-7,8-dihydro-2′-deoxyguanosine; AGA: α-1-acid glycoprotein; TNF-α: Tumour necrosis factor α; TNFR 1/2: Tumour necrosis factor α receptors 1 and 2; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor; CKD: Chronic kidney disease; UAE: Urinary albumin excretion; MA: Microalbuminuria.

diabetes, even at the normoalbuminuric stage[109,110]. A significant increased urinary excretion of VEGF in micro- and macroalbuminuric type 1 diabetic patients has been demonstrated[110]. Work in type 2 diabetes demonstrated that urinary VEGF concentration increases with DN stage. This has not been demonstrated in plasma[109]. However, baseline serum VEGF level did appear to be predictive of subsequent DN in a follow up study of children with type 1 diabetes[111]. In addition, both serum and urinary VEGF levels have been shown to be elevated in patients with diabetic retinopathy, although the sensitivity of urinary detection was poor[112]. Taken together, these findings led to the proposal that plasma VEGF is a reliable marker of generalised vascular dysfunction and retinopathy, whereas urinary concentration may serve as a sensitive predictor of risk of subsequent MA[109] (Table 1).

GENETIC FACTORS

In 1989 Seaquist *et al*[113] demonstrated strong familial clustering of DN, triggering a search for associated genetic variants. However, identifying gene variants that predispose to DN is complex as susceptibility is likely to be determined by a large number of common allelic variants, each of which may confer a modest increase in relative risk. In addition, overall risk of developing DN is a result of a combination of both genetic and environmental influences. Advances in genotyping technology have led to use of genome wide association scans (GWAS) for studying disease susceptibility across the entire genome. In relation to DN the creation of groups such as Family Investigation of Nephropathy and Diabetes (FIND) and Genetics of Kidneys in Diabetes (GoKinD) have facilitated such research.

The FIND group is a large multicentre consortium making use of family based linkage analyses in multiethnic groups to identify genes with significance in type 2 DN[114]. Results of the group's preliminary genome scan observed evidence linking chromosome loci 7q21.3, 10p15, 14q23.1 and 18q22.3 with DN[115]. Further publications by the group have shown a significant contribution of chromosomes 1q43, 8q13.3 and 18q23.3 to eGFR phenotype[116], and suggested contribution of chromosomes 3p, 7q, 16q and 22q to UAE status in African-American and European-American populations[117].

GoKinD group have accumulated a collection of DNA for genetic association studies of DN in the context of type 1 diabetes[118]. This group have identified genetic associations for DN susceptibility at candidate loci near the *FRMD3* and *CARS* genes[119]. In addition, variants in the *ELMO1* gene on chromosome 7p have previously been linked with DN in Japanese and African-American populations with type 2 diabetes[120]. GWAS data from the GoKinD collection confirmed this association in a Caucasian population[121].

A genome wide linkage scan in Diabetes Heart Study families detected significant evidence for linkage with eGFR on chromosomes 2p16, 7q21 and 13q13. Evidence for linkage to UAE however was far weaker[122]. In addition, genome wide DNA methylation analysis in a case control study of 192 Irish patients with type 1 diabetes identified 19 prospective CpG sites associated with risk of DN[123]. In 2012 the Genetics of Nephropathy: an International Effort consortium undertook a meta-analysis

of GWAS of DN in type 1 diabetes. They identified signals in an intron in the *AFF3* gene on chromosome 15 and linked this to DN mechanistically by providing evidence that AFF3 expression is linked to transforming growth factor beta-driven fibrosis in cultured epithelial cells[124,125]. Although this locus technically did not replicate, the potential for misclassification through identifying cases using clinical rather than histological criteria may have led to reduced statistical power[124].

PROTEOMICS

Proteomics is the study of the proteome, reflecting the protein content of the genome, and is defined as "the knowledge of the structure, function and expression of all proteins in the biochemical or biological context of organisms"[126]. These methods have attracted attention in recent years as a potentially important tool for early, pre-clinical disease detection as they allow simultaneous examination of the patterns of multiple urinary and plasma proteins. In view of the complex pathogenesis of type 2 diabetes, it is perhaps simplistic to expect that a single biomarker will provide sufficient sensitivity and specificity for disease prediction, detection and treatment monitoring, and therefore such multimarker approaches are appealing. Both urinary and plasma proteome analysis have identified a number of biomarkers which are significantly associated with DN, such as specific collagen fragments[127,128], cytokines[128,129] and RBP[130].

A panel of 65 urinary biomarkers (DN65) have been identified which distinguished normoalbuminuric patients with diabetes from those with DN. This panel proved sensitive and specific for distinguishing DN from other causes of CKD in both single and multicentre settings[127,131]. CKD273 is a panel of 273 urinary peptides which shows promise as a tool for early detection of DN. First described in 2010, the panel was initially shown to distinguish between CKD of any aetiology and healthy controls with 85.5% sensitivity and 100% specificity[132]. It has also recently been shown to predict adverse outcomes including death or end-stage renal disease in CKD patients[133]. Two further studies have demonstrated the predictive power of CKD273 in identifying diabetic patients at risk of progression to overt DN. In longitudinal samples from a small cohort of 35 diabetic patients Zürbig *et al*[134] showed that application of the classifier to samples from normoalbuminuric subjects up to 5 years prior to detection of macroalbuminuria enabled early identification of those at risk of progression (area under the curve 0.93, compared to 0.67 for urinary albumin). Similarly, Roscioni *et al*[135] applied the classifier to samples from the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort. They compared samples at baseline and 3 years for 44 "progressors" who transitioned from normo-to MA or from micro- to macroalbuminuria to matched controls who did not transition in albuminuria status. Results showed that classifier score at baseline was independently associated with progression of albuminuria[135]. Further to this CKD273 has recently been validated in a multicentre setting. In 165 urine samples obtained from 87 cases of DN and 78 controls at 9 centres worldwide the classifier distinguished cases from controls with high consistency across all centres (areas under the curve ranging from 0.95 to 1.00)[131]. A classification factor cut-off of 0.343 was established in the biomarker discovery cohort to highlight individuals "at risk" of later DN[132] and this has been confirmed by other studies[134,135].

METABOLOMICS

Metabolomics involves the measurement of low molecular weight intermediate and end-products of cellular functions in a biological sample, and has recently emerged as a tool with potential in novel biomarker discovery. The metabolome combines biological information from the genome, transcriptome and proteome, allowing identification of physiological and pathological changes in response to disease processes.As with proteomics, a variety of sample types including serum, plasma, tissue and urine can be analysed in this way[136].

A number of studies have explored the application of metabolomics approaches in kidney disease[136]. For example, in a cross sectional analysis of plasma metabolites using samples from 30 non-diabetic male subjects with CKD stage 2-4, major differences were identified in arginine metabolism, carboxylate anion transport and coagulation pathways with increasing CKD stage[137]. However, this study did not include patients with diabetes and in fact there are a limited number of such studies focussing on diabetic kidney disease. In serum samples from 78 type 2 diabetic participants, a panel of 19 metabolites was identified which could differentiate DN from normoalbuminuria, all of which correlated significantly with albumin creatinine ratio. A model comprising the five best performing markers (including γ-butyrobetaine and symmetric dimethylarginine) resulted in AUC value of 0.927 for diagnosis of DN[138]. Another study using serum samples from patients with DN, normoalbuminuric diabetic patients and healthy volunteers showed significant changes in amino acid and phospholipid metabolism between study categories, as evidenced by alterations in leucine, as well as the sphingolipids dihydrosphingosine and phytosphingosine[139]. Additionally, the application of metabolomics methods to renal cortex samples from streptozocin induced diabetic rats identified an increase in intrarenal organic toxins, including glucuronides, uraemic toxins and others associated with glucotoxicity, which were significantly correlated with 24 h urinary protein levels.Furthermore, treatment with the ACE-inhibitor Fosinopril appeared to block the accumulation of these toxins[140]. There is little published evidence from longitudinal studies to determine the predictive power of these methods for detection of individuals at risk of DN. One such paper published earlier this year described the application of metabolomics methods to urine and plasma samples from the PREVEND study over a median follow up period of 2.9 years. Differences were seen in plasma histidine

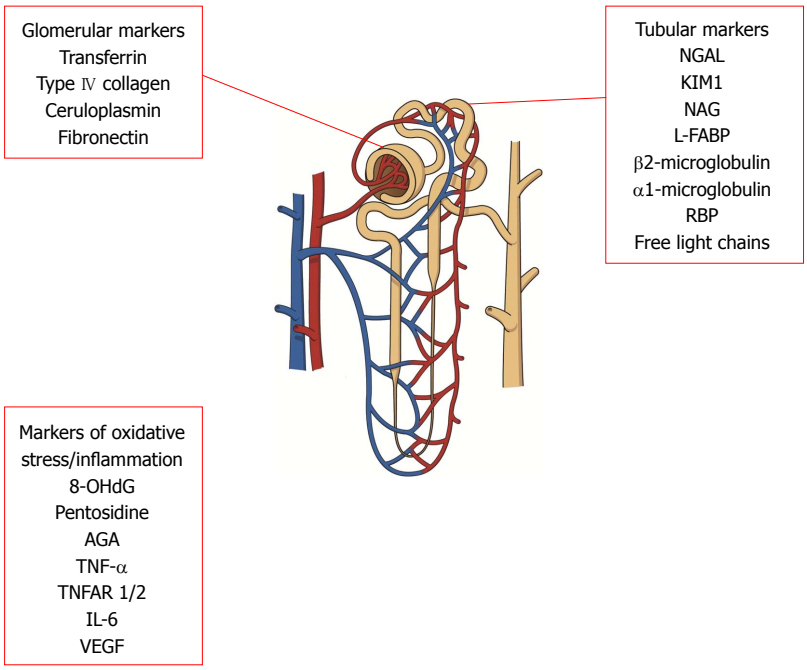

Figure 1 Biomarkers for diabetic nephropathy. NGAL: Neutrophil gelatinase associated lipocalin; KIM1: Kidney injury molecule 1; NAG: N-acetyl-b-d-glucosaminidase; L-FABP: Liver-type fatty acid binding protein; RBP: Retinol binding protein; 8-OHdG: 8-oxo-7,8-dihydro-2'-deoxyguanosine; AGA: α-1-acid glycoprotein; TNFAR 1/2: Tumor necrosis factors-α receptors 1 and 2; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor.

and butenoylcarnitine, as well as urine hexose, glutamine and tyrosine between individuals who transitioned in albuminuria stage compared to control sample who did not. Adding these metabolites to a predictive model including baseline albuminuria and eGFR appeared to improve risk estimation for transition to macroalbuminuria[141]. However, the complexity of the human metabolome remains perhaps the biggest challenge in translating these techniques into everyday clinical practice (Figure 1).

DISCUSSION AND CONCLUSIONS

DN is a leading cause of end stage renal disease and in combination with the increasing worldwide prevalence of diabetes poses an enormous burden to healthcare systems. UAE is currently the gold standard for detection and monitoring of nephropathy and cardiovascular risk in diabetes; however its predictive powers have limitations and research is focussing on biomarkers which may offer greater sensitivity and earlier detection to facilitate earlier intervention. A degree of caution should, however, be exercised in relation to aggressive early intervention as to date there is little evidence of benefit from these strategies and more intensive RAAS blockade can result in a high incidence of unwanted adverse effects[142,143]. The Randomised Olmesartan and Diabetes MA Prevention study confirmed a significant delay in onset of MA with olmesartan therapy in normoalbuminuric type 2 diabetes patients, but caused controversy regarding increased fatal cardiovascular events in the treatment group[144]. It could be argued that perhaps these studies have not targeted recruitment towards a population at particularly high risk of developing DN and focussing efforts in the direction of these individuals may yield more positive results. Identification of biomarkers to stratify patients according to DN risk may allow randomised controlled trials to focus on the population most likely to derive benefit from early, aggressive intervention.

Markers of glomerular damage show some promise for this purpose. In particular transferrin and type Ⅳ collagen appear to detect glomerular dysfunction at the normoalbuminuric stage although head to head comparative data are lacking. Similarly, given that tubular damage can precede glomerular pathology, markers such as NAG, KIM1 and NGAL are interesting. Evidence also points towards the role of oxidative stress in the pathogenesis of DN, meaning markers such as 8-OHdG and pentosidine merit further investigation. Low grade inflammation and endothelial damage is detectable in the pre-clinical stages of DN, leading to heightened interest in markers such as cytokines and AGA. These too appear to be potentially useful tools in the earlier detection of DN, although again comparative work in relation to UAE would strengthen the case for their use.

The development of new technologies has led to exciting possibilities in the search for ideal biomarkers for DN but, despite the vast number that have been studied, none has so far demonstrated superiority to albuminuria. While biomarker research in the preclinical setting is advancing, none of those biomarkers described above have been validated or are available commercially for clinical use. In addition, none have been described in relation to nonalbuminuric DN, which may reflect a separate disease process. All such potentially interesting markers require further large scale validation in prospective clinical studies to determine whether they can make the transition

from bench to bedside. Projects such as the EU-funded Proteomic prediction and Renin angiotensin aldosterone system Inhibition prevention Of early diabetic nephropathy In type 2 diabetic patients with normoalbuminuria (www.eu-priority.org) study which is currently recruiting, may help to redress this balance.

As the complexities of the biochemical mechanisms underpinning DN continue to be unravelled it is perhaps simplistic to expect that a single biomarker will be sufficient for risk stratification as we move towards predictive and personalised medicine, and as such the shift towards systems biology integrating different technologies into multimarker strategies might provide greater sensitivity and specificity.

PERSPECTIVES

A number of biomarkers show promise as tools for early detection of DN, yet to date none have out-performed microalbumin in larger scale, prospective longitudinal studies. Multimarker approaches such as metabolomic or proteomic methods are particularly appealing as they also offer an insight into the multiple complex pathophysiological processes underlying DN. In order to advance these efforts, cross-omics profiling, large scale biobanking and extended clinical phenotyping will be necessary to derive disease-stage specific models. It should be borne in mind that nonalbuminuric DN is not uncommon and may reflect an alternative underlying disease process, therefore longitudinal studies investigating the performance of biomarkers to identify these individuals early may also be of interest.

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P- Reviewer: Schuurman HJ, Said SAM **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Association of genetic variants with diabetic nephropathy

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Received: June 5, 2014 Revised: July 9, 2014
Accepted: September 6, 2014
Published online: December 15, 2014

Abstract

Diabetic nephropathy accounts for the most serious microvascular complication of diabetes mellitus. It is suggested that the prevalence of diabetic nephropathy will continue to increase in future posing a major challenge to the healthcare system resulting in increased morbidity and mortality. It occurs as a result of interaction between both genetic and environmental factors in individuals with both type 1 and type 2 diabetes. Genetic susceptibility has been proposed as an important factor for the development and progression of diabetic nephropathy, and various research efforts are being executed worldwide to identify the susceptibility gene for diabetic nephropathy. Numerous single nucleotide polymorphisms have been found in various genes giving rise to various gene variants which have been found to play a major role in genetic susceptibility to diabetic nephropathy. The risk of developing diabetic nephropathy is increased several times by inheriting risk alleles at susceptibility loci of various genes like *ACE*, *IL*, *TNF-α*, *COL4A1*, *eNOS*, *SOD2*, *APOE*, *GLUT*, *etc*. The identification of these genetic variants at a biomarker level could thus, allow the detection of those individuals at high risk for diabetic nephropathy which could thus help in the treatment, diagnosis and early prevention of the disease. The present review discusses about the various gene variants found till date to be associated with diabetic nephropathy.

Key words: Diabetes mellitus; Diabetic nephropathy; Genetic polymorphism; Gene variants; Nephropathy

Core tip: Diabetic nephropathy is actually the most common cause of kidney failure. It is now a scientifically proven fact that there is a strong association between an individual's genetic makeup in his predisposition to diabetic nephropathy. Multiple genes are involved in pathogenesis of diabetic nephropathy, with several allelic polymorphisms having demonstrable effects in the development and progression of the disease thus contributing to the overall risk. These gene polymorphism studies are thus conducted to identify at-risk patients and design therapeutic strategies to prevent the outcome of such complication in his later future. This review discusses about the various gene variants found till date to be associated with diabetic nephropathy.

Original sources: Rizvi S, Raza ST, Mahdi F. Association of genetic variants with diabetic nephropathy. *World J Diabetes* 2014; 5(6): 809-816 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/809.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.809

INTRODUCTION

Diabetes mellitus is a complex syndrome leading to various metabolic dysfunctions. These metabolic dysfunctions manifest characteristic long-term complications in the form of various microvascular diseases, including diabetic nephropathy, retinopathy, and neuropathy. Diabetic nephropathy is one of the major secondary complications of diabetes mellitus affecting almost 40% of the diabetic patients. Diabetic nephropathy is clinically characterized by proteinuria, declining glomerular filtration rate, hypertension eventually leading to renal failure, requiring dialysis or transplantation. Various risk factors like, hyperglycemia, increased blood pressure, and genetic

alterations may predispose an individual to diabetic nephropathy in the near future[1]. It is now a scientifically proven fact that apart from the above risk factors, there is a strong association between an individual's genetic make-up in his predisposition to diabetic nephropathy. In this context, Andersen *et al*[2] have shown that 35% of the patients with diabetes develop nephropathy, irrespective of glycemic control. Identification of genetic components of diabetic nephropathy is the most important area of diabetes research because elucidation of genes (alleles) associated with diabetic nephropathy will influence all efforts toward an understanding of the disease at molecular and mechanistic levels, its related complications, cure, treatment and prevention. Association studies of candidate genes for diabetic nephropathy are being conducted all around the globe to identify the biomarkers genes which may predispose a diabetic individual to the risk of diabetic nephropathy. Among the genetic factors involved, single nucleotide polymorphisms in the genes associated with diabetic nephropathy was found to have a major impact on the disease outcome. These gene polymorphism studies are thus conducted to identify at-risk patients and design therapeutic strategies to prevent the outcome of such complication in his later future.

GENE VARIANTS ASSOCIATED WITH DIABETIC NEPHROPATHY

It is now a scientifically proven fact that genes are amongst the major contributors to diabetic nephropathy apart from the environmental factors involved. In this context, a wide range of genes have been assessed to see their association with diabetic nephropathy along with a number of single-nucleotide polymorphisms in diabetic nephropathy susceptibility genes[3]. It is seen that different ethnic groups may have variable risk associated with a specific gene in individuals suffering from a particular disease like diabetic nephropathy. Given below is a discussion of few genes involved with diabetic nephropathy.

Inflammatory cytokines gene variants

Inflammatory cytokines are involved in pathogenesis of diabetic nephropathy and the genetic variability in the genes encoding these cytokines may predispose a person to diabetic nephropathy. Some of the cytokine gene variants found to be associated with diabetic nephropathy are as below.

Interleukins: There is a significant association between carriage of interleukins (IL)-1β allele 2 (-511 C/T polymorphism) and IL-1RN (IL-1 receptor Antagonist gene) allele 2 (2 copies of the repeat sequence) with diabetic nephropathy. In case of *IL-6* gene, C/G polymorphism at position 634 in the promoter region of the *IL-6* gene is a susceptibility factor for the progression of diabetic nephropathy where G/G homozygote showed a significant positive association with macroalbuminuria in type 2 diabetic patients from Japan[4]. In another study, Wang *et al*[5] identified a new amino acid change (V385I) that is associated with type 2 diabetic nephropathy. In case of IL-10, polymorphism (-592) in promoter region influence IL-10 and MCP-1 production, which may be an indicator of type 2 diabetic nephropathy risk in Taiwanese patients[6].

Tumour necrosis factor: Gene for tumour necrosis factor (*TNF*)-α is highly polymorphic and is located on chromosome 6p. *TNF*-α -308G/A polymorphism has been implicated in susceptibility to diabetic nephropathy but the results have been contradictory. Studies have shown that polymorphism of the TNF-α gene at the -308 position is significantly related to an increased risk of kidney failure in patients with type 2 diabetes (T2DM)[7,8]. In contrast to this, Lindholm *et al*[9], demonstrated that the allele frequencies of *TNF* -308 G→A and *LTA* T60N polymorphisms were similar in type 1 diabetic patients with and without diabetic nephropathy and no differences were observed between type 2 diabetic patients with and without diabetic nephropathy in allele or haplotype frequencies of the studied polymorphisms. In a recent meta analysis it was demonstrated that A allele of *TNF*-α -308G/A polymorphism might be protective against diabetic nephropathy but with ethnic selectivity[10].

Genetic variants of extracellular matrix components

Collagen, type Ⅳ, alpha 1: The Collagen, type Ⅳ, alpha 1 (*COL4A1*) provides instructions for making one component of type Ⅳ collagen, which is a flexible protein important in the structure of many tissues throughout the body. Two single nucleotide polymorphism's in intron 1 (rs614282 and rs679062) showed significant association with diabetic nephropathy[3]. Other studies on genetic variants of *COL4A1* gene have shown contradictory results where Krolewski *et al*[11] showed that a polymorphic *Hin*dⅢ restriction site was associated with increased risk for progression to diabetic nephropathy and contradictory to it, Chen *et al*[12] found no association in larger sample size.

Laminins: Laminins (LAM) are extracellular matrix glycoproteins which are the major noncollagenous constituent of basement membranes. They are involved in various biological processes like cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis. Ewens *et al*[3] found a gene variant (rs3734287) located in *LAMA4* gene's intronic region and Asn837Asn variant (rs20557) in *LAMC1* gene, to be significantly associated with diabetic nephropathy.

Matrix metalloproteinase 9: Two studies conducted by Maeda *et al*[13] and Hirakawa *et al*[14] had found evidence for association between diabetic nephropathy and Short Tandem-Repeat Polymorphism in the promoter microsatellite locus (D20S838) of Matrix metalloproteinase 9 (*MMP9*) in Japanese and Caucasian type 2 diabetic patients, respectively. In contrast, Ewens *et al*[3], found no evidence of association between any D20S838 allele with

diabetic nephropathy. However, significant association was seen between diabetic nephropathy and rs11697325, an SNP located 8.2 kb 5' of *MMP9*[13,14].

Gene variants of renal function components

Angiotensin I -converting enzyme: Angiotensin-converting enzyme is a potent vaso-constrictor and increases blood pressure. Polymorphisms in this gene are clearly associated with circulating angiotensin I -converting enzyme (ACE) levels and studies have shown positive association between the *ACE* DD allele and type 1 diabetic nephropathy[15-17]. This study is in confirmation to a meta analysis where subjects with the II genotype had a 22% lower risk of diabetic nephropathy than carriers of the D allele suggesting a genetic association of the *ACE* I /D polymorphism with diabetic nephropathy in type I[18] and type II patients[19]. Although a large meta-analysis failed to confirm the diabetic nephropathy association in white individuals[20] but another report from the European Rational Approach for the Genetics of Diabetic Complications (EURAGEDIC) Study Group detected evidence for association of several *ACE* polymorphisms (including the "D" deletion allele) in a large case-control study, with somewhat consistent findings in a family-based transmission disequilibrium testing analysis[15]. A study on Iranian population also showed similar results where neither the DD genotype nor the D allele was associated with diabetic nephropathy[21].

Angiotensinogen and angiotensin II receptor type 1 and 2 (AGT and AGTR1, AT2R): A meta-analysis conducted by Mooyaart *et al*[22], found no association between gene variants in the renin-angiotensin system, such as the rs699 variant of angiotensinogen (*AGT*) and the rs5186 polymorphism of angiotensin II receptor type 1 (*AGTR1*), with diabetic nephropathy. In contrast, a recent study on angiotensin type 2 receptor (*AT2R*) found an association between the AT2R -1332 G:A polymorphism and the risk of diabetic nephropathy in females[23].

Gene variants of endothelial function and oxidative stress

Nitric oxide synthase 3 (NOS): It is considered as a potential candidate gene for diabetic nephropathy susceptibility[24,25]. Three polymorphisms in this gene *G894T* missense mutation (rs1799983), a 27-bp repeat in intron 4, and the T786C single nucleotide polymorphism (SNP) in the promoter (rs2070744) have been found to be associated with diabetic nephropathy susceptibility[26-30].

The G894T variant was found to increase the risk of macroalbuminuria and progression from microalbuminuria to macroalbuminuria, with declining glomerular filtration rate as serum creatinine value rises progressively, culminating in nephropathy[31,32] However, these results have been contradictory and not all studies support this association[33-35]. Recent studies on different gene variants observed that there was an association between *eNOS*-4b/a polymorphism and the risk of type 2 diabetic nephropathy[36,37] while others suggested that there was no significant association[38]. Recently, a report from Arab population also failed to find an association between *eNOS* gene G894T polymorphism with the risk of type 2 diabetic nephropathy[39].

Catalase: This enzyme protects the cell from oxidative damage by reactive oxygen species (ROS) by breaking down hydrogen peroxide to water and oxygen. Two variants of catalase (*CAT*) gene one located in the 5'-untranslated region (rs1049982) and other located in intron 1 (rs560807) were found to be involved with the risk of type 1 diabetic nephropathy[3].

Superoxide dismutase 2 (MnSOD/SOD2): Manganese superoxide dismutase (MnSOD) protects the cells from oxidative damage by scavenging free radicals. The study on valine/alanine polymorphism in *MnSOD* gene (V16A, rs4880) revealed that, the subjects with Val allele were associated with increased risk of type 1 diabetic nephropathy[40]. The result of this study is in agreement with results by other studies[41,42], who found lower frequency of the Ala allele in Japanese and Korean type 2 diabetic patients with diabetic nephropathy as compared to controls. This Val allele was more common in the Japanese and Korean populations (85%-90%) than the northern Caucasian population (50%) and is strongly associated with diabetic nephropathy. A recent study showed that *SOD2* Val16Ala polymorphism was significantly associated with macroalbuminuria in a sample of Mexican type 2 diabetes patients where the frequency of the TT genotype was 6.7% higher in participants with macroalbuminuria than in the normoalbuminuria group[43].

Gene variants of glucose and lipid metabolism

Adiponectin (ADIPO): It is a adipocytokine encoded by adiponectin gene with substantial anti-inflammatory properties and is a major modulator of insulin resistance and dyslipidemia. The minor allele (A) in intron 1 (rs182052) of adiponectin gene was found to be associated with diabetic nephropathy in an African American population[44]. Another study showed the strongest association between a polymorphism in the promoter region of adiponectin gene, rs17300539 (*ADIPOQ*_prom2/rs17300539 G > A) and diabetic nephropathy where the A-allele was found to increase the risk for nephropathy while the G-allele was found to be protective against the same. This association was found to be significant in Denmark and marginal in France but was not significant in Finland[45]. However, in a study conducted by Mooyaart *et al*[22], found no link between rs17300539 of adiponectin gene with diabetic nephropathy.

Apolipoprotein E: The apolipoprotein gene has been found to be associated with increased susceptibility to diabetic nephropathy[46]. It is a triallelic gene consisting of ε2, ε3, and ε4 alleles which are defined by a single amino acid substitution at two sites[47]. Amongst these alleles, E2

and the E4 allele of apolipoprotein E (*APOE*) gene were found to be associated with diabetic nephropathy in a meta-analysis[22] where, E2 allele lead to an increased risk of diabetic nephropathy and the E4 allele was found to have a protective effect. However, the influence of three-allelic variations in the *APOE* gene for the development of diabetic nephropathy may be weak or moderate, but not strong[48].

Aldose reductase: This enzyme catalyzes the reduction of glucose to sorbitol in the first step in polyol pathway of glucose metabolism. Ko *et al*[49] first identified seven alleles at the locus of the (AC)n dinucleotide repeat sequence upstream of Aldose reductase gene (*AKR1B1*). Several studies have demonstrated a correlation between the Z-2 allele (23 AC repeats) and susceptibility to an increased risk of diabetic nephropathy in both type1 and type 2 diabetes mellitus[50,51]. Heesom *et al*[52] also showed that individuals with the Z+2 allele are more than seven times less likely to develop diabetic nephropathy than those without this gene variant. A meta-analysis found a correlation between the (AC)n dinucleotide repeat polymorphism and the occurrence of diabetic nephropathy in Caucasian type 1 diabetic subjects in contrast to type 2 diabetic subject population in which neither the risk ZK2 allele nor the protective ZC2 allele in type 1 diabetic subjects appeared to have an effect on nephropathy in type 2 diabetic subjects[53]. A second polymorphism in this gene has been observed at position-106 of its promoter region. This polymorphism in aldose reductase gene was also found to be associated with nephropathy in type 1 and type 2 diabetic patients[54]. This polymorphism was also found to be involved in the early development of microalbuminuria in Finnish T2DM patients and was proposed as a risk factor for development of nephropathy in T2DM patients with poor glycaemic control[55].

Glucose transporter 1: Glucose transporter 1 (*GLUT1 or SLC2A1*) is the major facilitative glucose transporter in glomerular mesangial cells. Experimental evidence suggests that *GLUT1* may be associated with hypertensive glomerulopathy[56]. Ng *et al*[57], showed that SNPs at the *GLUT1* (XbaI -intron 2 and HaeIII SNPs-exon 2) were associated with susceptibility to diabetic nephropathy in type 1 diabetes. A meta-analysis on the other hand demonstrated a significant association between the another polymorphic site *SLC2A1* XbaI in *GLUT1* gene with Diabetic nephropathy[58].

A study of those with type 1 diabetes examined six *GLUT1* SNPs and found homozygosity for the XBAI A allele and for minor allele(C-to-T) of the enhancer-2 SNP1 (ENH2 SNP) was associated with diabetic nephropathy in type 1 diabetes[57] whereas, no statistically significant association was found between *Xba* I gene variants and type 2 diabetic nephropathy[57]. Among the gene variants identified in the *GLUT1* putative enhancer elements, the AA genotype of enhancer-2 SNP1 (rs841847) is a "risk genotype"[57] and that the TT genotype of the 5' promoter region (rs710218) was associated with nephropathy[59]. Moreover, the patients with the AG haplotype (rs841847-rs841853) have an increased risk of diabetic nephropathy and the TT haplotype (rs710218-rs841853) was more frequent in nephropathic patients. These findings showed that two haplotypes (composed of rs1385129-rs841847-rs841848) are associated with a 4.4 and 2.6-fold increased risk of nephropathy in the Tunisian T2DM patients[60].

However, the results of various case-control studies on *GLUT1* gene variants and their association with diabetic nephropathy have been inconsistent showing heterogeneity between studies[57,61-63].

Peroxisome proliferator-activated receptor gamma 2: Peroxisome proliferator-activated receptor gamma 2 (PPARG2) is a receptor expressed selectively in the adipose tissue where it modulates the expression of genes involved in adipocyte differentiation and glucose homeostasis. The *Pro12Ala* gene variant was associated with lower albumin excretion rates among Ala12 carriers with type 2 diabetic nephropathy. Thus it could be suggested that Pro12Ala polymorphism may be protective against the disease since microalbuminuria is considered to be a risk factor for diabetic nephropathy[64]. This study was confirmed by Pollex *et al*[65] who showed that the Ala12 allele carriers have 1.5-fold reduction of the albumin/creatinine ratio and thus reduced occurrence of microalbuminuria. A recent meta-analysis showed that Pro12Ala polymorphism in *PPARγ2* gene is not a risk factor for diabetic nephropathy in type 2 diabetes[66].

Other gene variants involved

Apart from the above mentioned genes and their variants, there are various other gene variants for various genes like genes coding for growth factor, inflammatory factors, transcription factors, cytoskeletal proteins, components of immune system etc which have also been implicated in predisposing an individual to the risk of developing diabetic nephropathy. Some of these gene variants are discussed in Table 1.

CONCLUSION

Diabetic nephropathy is progressively becoming a major challenge for the health care system, since it is as yet poorly understood in many aspects. It is the leading cause of premature death in young diabetic patients (between 50 and 70 years old). It is a heterogenous and a multifactorial disease with several genes, proteins and environmental factors contributing to its risk. Due to the growing burden of the disease in diabetic patients, it is important to identify diabetic nephropathy predictors, for the proper management of this disease. Genetic susceptibility has been proposed as an important factor for diabetic nephropathy. Multiple genes are involved in pathogenesis of diabetic nephropathy, with several allelic polymorphisms having demonstrable effects in the devel-

Table 1 Gene variants associated with diabetic nephropathy

Gene category	Gene name	Gene variant symbol	Location	Phenotype	Ref.
Growth factors	Insulin-like growth factor 1	IGF-1	12q23.2	Type 1 DN	[3]
	IGF-binding protein 1	IGFBP1	7p14	Type 2 DN	[67]
	Transforming growth factor-β receptor Ⅱ	TGF β R2	3p24.1	Type 1 DN	[3]
	TGF-β receptor Ⅲ	TGF β R3	1p22.1	Type 1 DN	[3]
Matrix metalloproteinases and dipeptidases	Tissue inhibitor of metalloproteinase 3	TIMP3	22q12.3	Type 1 DN	[3]
	Matrix metalloproteinase 9	MMP9	20q13.12	Type 1 DN	[3]
	Carnosinase	CNDP1	18q22.3	Type 2 DN	[68,69]
Transcription factors	Transcription factor 2, hepatic	HNF1B1/TCF2	17q12	Type 1 DN	[3]
	Neuropilin 1	NRPI	10p11.22	Type 1 DN	[3]
	Protein kinase C β 1	PRKCBI	16p12.1	Type 1 DN	[3]
	Upstream transcription factor 1	USFI	1q23.3	Type 1 DN	[3]
Other genes	Engulfment and cell motility factor	ELMO1	7p14	Type 2 DN	[70-72]
	Cytochrome b, α polypeptide	p22phox	16q24.3	Type 1 DN	[3]
	Glutathione peroxidase 1	GPXI	3p21.3	Type 1 DN	[3]
	B-cell leukemia/lymphoma 2 (bcl-2)	BCL2	18q21.33	Type 1 DN	[3]
	Aquaporin 1	AQP1	7p14.3	Type 1 DN	[3]

opment and progression of the disease thus contributing to the overall risk. These polymorphisms in several genes distributed widely across the human genome, each with a modest effect size, may be causal or protective factors in the development and progression of diabetic nephropathy. The combining of the various gene polymorphism studies in diabetic nephropathy related genes with recent researches/developments in the fields of human genomics, proteomics and bioinformatics would help in early diagnosis, treatment and prevention by giving us a better understanding of the pathogenesis of diabetic nephropathy. Identification of genes associated with diabetic nephropathy could provide a powerful tool for identifying patients at risk of developing diabetic nephropathy in the late future. In this context research efforts have been invested worldwide to identify the susceptibility gene for diabetic nephropathy. Epidemiologic studies and candidate-gene-based association studies are the most common approaches employed to identify susceptibility genes for diabetic nephropathy. Many genes were found to be associated with the disease but the results had been inconsistent and most of the candidate genes for diabetic nephropathy remain still to be identified. The inclusion of genetic studies in design and analysis of drug trials could lead to development of genetic biomarkers that predict treatment response. Thus, collaborative efforts are needed to achieve substantial findings in the study of genetics of diabetic nephropathy which could give us a better prospective of biochemical and molecular mechanism of disease on the whole. Early identification of at risk patients will facilitate earlier intervention; ultimately delaying and reducing the impact of nephropathy remain still to be identified. Thus, collaborative efforts are needed to achieve substantial.

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P- Reviewer: Gao C, Yong D, Yorioka N **S- Editor**: Song XX
L- Editor: A **E- Editor**: Lu YJ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (1): Insulin

Defect of insulin signal in peripheral tissues: Important role of ceramide

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Supported by INSERM, the Société Francophone du Diabète and from an Agence Nationale de la Recherche grant project (Crisalis)
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Received: December 12, 2013 Revised: January 29, 2014
Accepted: May 8, 2014
Published online: June 15, 2014

Abstract

In healthy people, balance between glucose production and its utilization is precisely controlled. When circulating glucose reaches a critical threshold level, pancreatic β cells secrete insulin that has two major actions: to lower circulating glucose levels by facilitating its uptake mainly into skeletal muscle while inhibiting its production by the liver. Interestingly, dietary triglycerides are the main source of fatty acids to fulfill energy needs of oxidative tissues. Normally, the unconsumed fraction of excess of fatty acids is stored in lipid droplets that are localized in adipocytes to provide energy during fasting periods. Thus, adipose tissue acts as a trap for fatty acid excess liberated from plasma triglycerides. When the buffering action of adipose tissue to store fatty acids is impaired, fatty acids that build up in other tissues are metabolized as sphingolipid derivatives such as ceramides. Several studies suggest that ceramides are among the most active lipid second messengers to inhibit the insulin signaling pathway and this review describes the major role played by ceramide accumulation in the development of insulin resistance of peripherals tissues through the targeting of specific proteins of the insulin signaling pathway.

Key words: Diabetes; Insulin resistance; Lipids; Insulin signaling; Triglycerides; Palmitate; Sphingolipid; Akt; Ceramide synthase; Protein phosphatase 2A; Protein kinase C ζ/λ

Core tip: Muscle and liver represent major sites for insulin-mediated glucose metabolism. The ability of insulin to promote its peripheral action is reduced significantly by excess of saturated fat that stimulates intracellular production of second-messenger lipids such as ceramide. Studies suggest that ceramide could be important contributors to lipotoxicity, as the inhibition of early steps its biosynthesis pathway has large beneficial effects in rodent models of obesity and diabetes. In this review, we describe mechanisms by which ceramide acts on insulin-sensitive tissues and we propose that targeting this lipid family could be an interesting approach to fight diabetes.

Original sources: Hage Hassan R, Bourron O, Hajduch E. Defect of insulin signal in peripheral tissues: Important role of ceramide. *World J Diabetes* 2014; 5(3): 244-257 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/244.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.244

DIABETES EPIDEMIC

Diabetes has become a serious public health problem in

both developed and developing countries. Indeed, there is a dramatic increasing incidence of diabetes in most of these countries. In 2005, 217 million people worldwide had diabetes, and the World Health Organisation predicts that it will increase to 366 million in 2030[1]. In 2050, 33% of the population of the United States will suffer from diabetes[2]. One consequence is that over the years, diabetes has become life-threatening, with increased risk of cardiovascular diseases, retinopathy, kidney failure, and nerve and artery damages[3]. Diabetes is one of the first causes of haemodialysis, of blindness and of non-traumatic amputation of the legs. Another consequence is the increasing of health spending due to diabetes. For example, in the United States, diabetes costing is actually evaluated to more than $174 billion per year and it's expected to increase in subsequent years[2].

PATHOPHYSIOLOGY OF TYPE 2 DIABETES

There are different types of diabetes: (1) type 1 diabetes or maturity onset diabetes of the young associated to impairment of insulin production; and (2) type 2 diabetes, corresponding to 85%-90% of all diabetes, with both insulin secretion defects and peripheral insulin resistance. Type 2 diabetes is associated with obesity and although genetic factors play a role in the pathophysiology of this disease, other environmental factors such as diet and physical activity both play large roles. Several mechanisms have been proposed to explain both insulin resistance and insulin secretion defects observed in type 2 diabetes. Lipotoxicity, glucotoxicity, low grad systemic inflammation, oxidative stress and endoplasmic reticulum stress[4-6] correspond to different mechanisms that converge on a common pathway to induce insulin resistance. In this review we will focus on cellular lipid toxicity, *i.e.*, lipotoxicity.

LIPOTOXICITY

Systemic lipid imbalances are common in metabolic syndrome, in pre-diabetes and in type 2 diabetes and it is now clear that lipotoxicity can induce glucose dysregulation and participate to the pathophysiology of type 2 diabetes[7-9]. For example, prospective epidemiological studies performed in population with low or high risk to develop type 2 diabetes have shown that high free fatty acid (FFA) concentrations in plasma are associated with the risk of incident type 2 diabetes[10-12].

A major characteristic of type 2 diabetes is the loss of the ability of pancreatic β cells to increase insulin secretion to maintain normoglycemia in the face of insulin resistance[13]. Because of genetic predisposition, β cells could be unable to compensate the insulin resistance induced by FFA, but chronic exposition of β cells to high levels of FFA could equally explain defects in β cell function and decreased mass observed in type 2 diabetes. Indeed, *in vitro* studies have shown that FFA are associated with a decrease of insulin expression, synthesis and processing[14-16]. Another mechanism that can explain insulin secretion dysfunction in type 2 diabetes is that high FFA levels in islets induce β cell death[17]. In this review, we will not deal with this topic but we will rather focus our message on lipid-induced peripheral insulin resistance. To more information on lipotoxicity in pancreatic beta cells, confer to the excellent review of Boslem *et al*[18].

Since skeletal muscle constitutes 40% of human body mass and is quantitatively the most important tissue in regard to insulin-stimulated glucose disposal, it is considered the main cellular target in the development of insulin resistance. Thus, most of the studies investigating mechanisms of lipotoxicity induced insulin resistance were mostly performed in muscle tissue.

In 1963, Randle *et al*[19] have postulated that a competition between glucose and fatty acids for their oxidation and uptake is responsible for the onset of insulin resistance in muscle and adipose tissue. *In vivo* studies performed in both rodents and humans confirmed such insulin resistance obtained after lipid infusion but they also demonstrated that, in opposite to Randle's hypothesis, insulin resistance induced by lipids was not secondary to decreased glycolysis[20]. Indeed, lipids act directly on insulin signaling, resulting in an inhibition of the translocation of the insulin sensitive glucose transporter GLUT4 to the plasma membrane in response to the hormone, with subsequent reduced glucose uptake[21-25]. In human, data clearly show a strong correlation between lipid intramuscular content and insulin resistance[26-28] and a cross-sectional analysis performed in young, normal weight and non-diabetic adults reveals that a better correlation exists between muscle insulin sensitivity, assessed by the hyperinsulinaemic-euglycaemic clamp technique, and intramyocellular lipid content rather than with circulating lipid levels, body mass index, fasting blood glucose and age[29].

Liver is another important organ implicated in insulin resistance and, like in muscle indirect data also suggest an inverse relationship between lipid liver content and insulin sensibility. Indeed, ectopic lipid accumulation in the liver, termed nonalcoholic fatty liver disease (NAFLD), is associated with insulin resistance. Interestingly, in an animal model of lipodystrophy with steatosis, but without increased visceral fat, lipid liver content is associated with insulin resistance. Insulin resistance is reversed after reduction of steatosis with liver transplantation or recombinant leptin treatment[30]. Such association between steatosis and insulin resistance has also been observed in patients with severe lipodystrophy with equally a good response to recombinant leptin therapy[31]. Similarly, hepatic specific overexpression of lipoprotein lipase leads specifically to hepatic steatosis and hepatic insulin resistance[32,33]. During type 2 diabetes, reduction of steatosis by caloric restriction, or gastric bypass, is associated with increased insulin sensibility independently of visceral fat mass reduction[34,35].

Strong evidence exists between ectopic lipid accumulation and insulin resistance. However, in some cases, like in the "athlete's paradox", there is a lack of correlation

between ectopic lipid accumulation and peripheral insulin resistance. Indeed, athletes display high insulin sensitivity but also present increased levels of intramuscular fatty acids[36]. Thus, it seems that ectopic accumulation of fatty acids in non-adipose tissues can only be used as markers for the onset of insulin resistance but cannot be considered as a direct cause. Even if they do not seem to be directly involved, fatty acids contribute to insulin resistance as they lead to the synthesis of many lipid derivative intermediates such as diacylglycerol (DAG) and ceramide.

Over the years, studies have provided conclusive proof that ceramide plays a key role in the progression of insulin resistance in insulin sensitive tissues, targeting and inhibiting specific actors of the insulin signaling pathway.

INSULIN SIGNALING PATHWAY AND METABOLIC FUNCTIONS

Insulin is a polypeptide hormone whose major physiological role is to control glucose homeostasis by stimulating glucose uptake into insulin sensitive tissues (skeletal muscle and adipose tissue) and by inhibiting glucose output from the liver[37]. Insulin consists of two polypeptide chains, a α chain of 21 amino acid residues linked by two disulfide bonds to a β chain of 30 amino acid residues. Insulin is produced in the β cells of the Islets of Langerhans found in the pancreas. It is initially synthesized as an immature single polypeptide chain of 110 amino acids called pre-proinsulin. Pre-proinsulin contains an N-terminal domain of 24 amino acids that acts to direct the polypeptide to the endoplasmic reticulum during translation. This domain is later cleaved to yield proinsulin. Proinsulin is transported to the secretory vesicles of the pancreatic β cells, where a proteolytic enzyme removes the central 35 residues of the peptide (termed the C-peptide) that connect α and β chains to produce insulin. Insulin is then released into the blood stream by exocytosis. Secretion of the hormone is regulated by the glucose abundance in the plasma.

In skeletal muscle, insulin promotes the uptake of glucose and its conversion into glycogen. This tissue is an important target of the hormone, representing the major site of glucose disposal *in vivo*[37] and is reported to mediate 70%-80% of whole body insulin-stimulated glucose transport[38]. In the liver, insulin stimulates the synthesis of glycogen while inhibiting gluconeogenesis and glycogenolysis, halting hepatic glucose output. In adipocytes, insulin promotes the uptake of glucose and its conversion into a glycerophosphate of which can be esterified by 3 fatty acids, allowing to form triglycerides for long term storage, whereas simultaneously inhibiting the lipolytic pathway[39]. In addition to glucose metabolism, insulin also regulates many other cellular processes including amino acid transport, lipogenesis, protein synthesis and mitogenesis.

The first step in the activation of the insulin signaling pathway is the binding of insulin with its membrane receptor, the insulin receptor (IR). IR is a heterotetrameric complex of two subunits: α-subunit, and β-subunit that possess a transmembrane domain and an intracellular part. Binding of insulin to α subunits of IR induces a rapid conformational change in the receptor. This in turn stimulates the intrinsic tyrosine kinase activity of the β subunit resulting in trans-autophosphorylation of tyrosine residues in the intracellular region of the β subunits[40]. As a result of this autophosphorylation, the IR becomes catalytically active and promotes the tyrosine phosphorylation of a number of cellular proteins including the IR Substrate (IRS) proteins.

IRS proteins are major physiological targets of the activated insulin receptor kinase. Six different IRS isoforms have been identified so far[41]. In skeletal muscle and adipose tissue, IRS1 is the isoform that mediate insulin signaling. In the liver, however, IRS2 is the one that drives insulin metabolic functions. In the pancreas, IRS2 is an important regulator of cell growth and regeneration[41]. Studies have also shown that both IRS3 and IRS4 can be activated in response to insulin and insulin-like growth factor 1 (IGF1)[42] and that IRS3 can mediate insulin signaling in adipocytes[42]. Mice lacking either IRS3 or IRS4, however, display no major phenotype, suggesting that neither isoform plays a direct role in controlling glucose metabolism[43,44] but may rather act as negative regulators of the IGF1 signaling pathway by suppressing the function of other IRS isoforms[45].

One key molecule that is activated by the IRSs in response to insulin is phosphoinositide-3-kinase (PI3K). PI3K is a lipid kinase, which phosphorylates the D3 position of the inositol ring within inositol lipids resulting in the generation of 3-phosphoinositides (*e.g.*, PI-3P, PI-3,4P_2, and PI-3,4,5P_3). Eight mammalian isoforms of PI3K exist and they are grouped into three classes on the basis of their substrate specificity and structure: class Ⅰ, class Ⅱ, and class Ⅲ. Only class I can phosphorylate phosphatidylinositol, 4, 5-bisphosphate (PIP_2)[46]. Following PI3K activation, PIP_3 is generated from the substrate PIP_2. PIP_3 binds a protein displaying a PH domain and called the 3-phosphoinositide-dependent protein Kinase 1 (PDK1). Activated-PDK1 triggers downstream targets such as protein kinase B (PKB/Akt)[47].

PKB/Akt also called Akt is the third central node activated by insulin. It plays a crucial role in mediating signaling effects on metabolism, cell growth and cell cycle[48,49]. PKB/Akt has three isoforms: PKBα/Akt1, ubiquitously expressed, PKBβ/Akt2 mostly present in insulin responsive tissues (liver, adipose tissue and muscle), and PKBγ/Akt3 predominant in the brain. PKBβ/Akt2 is the isoform implicated in the regulation of glucose metabolism since neither PKBα Akt1 nor PKBγ/Akt3 ablation affects glucose metabolism[50].

PKB/Akt is activated through PI3K-produced PIP_3 which binds its PH domain. Then, PKB/Akt is recruited to the plasma membrane where it is activated by phosphorylation on two critical sites: threonine 308 (T308) in the activation loop and serine 473 (S473) in the hydrophobic motif[51]. PDK1 phosphorylates PKB/Akt on T308.

The kinase that phosphorylates the S473 site is the complex mammalian target of rapamycin complex 2, a regulator of cell growth and proliferation[52].

PKB/Akt is highly activated within minutes following cell exposure to insulin to mediate the metabolic effects of the hormone[49,53].

Indeed, principle roles of PKB/Akt in insulin sensitive tissues are to: (1) Stimulate glucose uptake in muscle and adipose tissue; (2) Trigger glucose storage as glycogen in muscle and in the liver; (3) Stimulate the conversion of glucose excess into lipids in the liver; (4) Induce protein synthesis in muscle; (5) Inhibit glycogen breakdown in both muscle and liver; (6) Suppress liberation of free fatty acids from adipose tissue; (7) Inhibit *de novo* production of glucose in the liver; and (8) Impede protein breakdown in muscle (Figure 1).

Considering the crucial role PKB/Akt plays in mediating insulin metabolic actions in cells, impairing PKB/Akt activity represents the best way to compromise the whole system.

LIPID SECOND MESSENGER AND LOSS OF INSULIN SENSITIVITY

In pathological situations such as obesity and type 2 diabetes that are characterized by insulin resistance, ectopic fatty acid accumulation is increased due to reduced mitochondrial fatty acid oxidation and to enhanced fatty acid uptake[54-57]. This increased fat content inversely correlates with insulin sensitivity in skeletal muscle, liver and adipocytes[58-61].

Interestingly and depending on the degree of saturation, free fatty acid may exert different effects on insulin signaling. Studies have demonstrated that saturated fatty acids such as palmitate (16:0) and stearate (18:0) impair insulin sensitivity in muscle[62,63], whereas mono-unsaturated fatty acids or poly-unsaturated fatty acids have no effect or even enhance insulin action[64-66]. Although the exact reasons behind these differences are unclear, studies have suggested that unsaturated fatty acids may be preferentially targeted for triglyceride synthesis and storage, whilst saturated fatty acids may be used for synthesis of critical lipid intermediates such as DAG and ceramide. These two lipid second messengers have been demonstrated to mediate deleterious actions of saturated fatty acids on insulin signaling.

DAG AND INSULIN RESISTANCE

DAG is a glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule. DAG, intermediate of both triglyceride and phospholipid metabolism, is an important second messenger involved in intracellular signaling[67].

DAG has been shown to accumulate in insulin resistant liver[68,69] and studies have shown that intra-hepatic DAG is an important mediator of hepatic insulin resistance in obese people with nonalcoholic fatty liver disease[70,71]. Elevated DAG content and activation of protein kinase C (PKC)ε has been associated with hepatic insulin resistance and the involvement of this "lipid-activated pathway" has been validated through the use of antisense oligonucleotide against PKCε in rats. Knocking down PKCε expression in liver protected rats from lipid-induced hepatic insulin resistance, despite increase in hepatic lipid content[72].

Several studies have decrypted the mechanism by which DAG-activated PKCs inhibit insulin signaling in liver. They show that IRS proteins are likely to be PKC's preferential targets. DAG-activated PKCs inhibit IRSs activity through their phosphorylation on several serine residues, preventing consequently insulin activation of IRSs through their phosphorylation on tyrosine residues[73-75].

In muscle, however, data are contradictory. Itani *et al*[76] were the first to point out the positive association between DAG content and muscle insulin resistance by comparing a group of subject receiving a lipid infusion to a control group. Lipid infusion resulted in a 3-fold increase in total DAG content in muscle, and reduced insulin sensitivity. Straczkowski *et al*[77] observed that total muscle DAG concentrations were higher in obese compared to lean controls and lean offspring type 2 diabetics, and this increased DAG content was inversely related to insulin sensitivity. Other studies have also confirmed this correlation[78,79].

However, the association between DAG and muscle insulin resistance is still questioned. Indeed, Vistisen *et al*[80] performed muscle biopsies during glucose clamps and they observed a reduction in insulin sensitivity after lipid infusion, without any changes in muscle DAG content. These results were confirmed by Anastasiou *et al*[81] that compared obese type 2 diabetic patients to non-diabetics subjects and found no difference in muscle DAG content between the groups. Similarly, Perreault *et al*[82] compared insulin resistant obese patients to glucose tolerant obese patients and again found no difference in DAG content between the groups. Even more intriguing, Amati *el al*[83] observed a two-fold increase in DAG content in insulin sensitive human muscle biopsies compared to insulin resistant human muscle biopsies. More recently, the same group showed no difference in muscle DAG content between lean subjects compared to obese insulin resistance patients[84].

Altogether, and in opposite to liver, it seems that DAG does not appear to be a crucial player in the onset of insulin resistance in muscle, and maybe more investigations are needed to really be able to conclude.

CERAMIDE AND INSULIN RESISTANCE

Ceramide biosynthesis

One of the main sphingolipid that has been demonstrated to play a crucial role in insulin resistance is ceramide. During obesity, ceramide is mainly generated from long chain fatty acyl-CoAs[85,86], and has been shown to be toxic lipid when it accumulates in tissues during obesity[87-89].

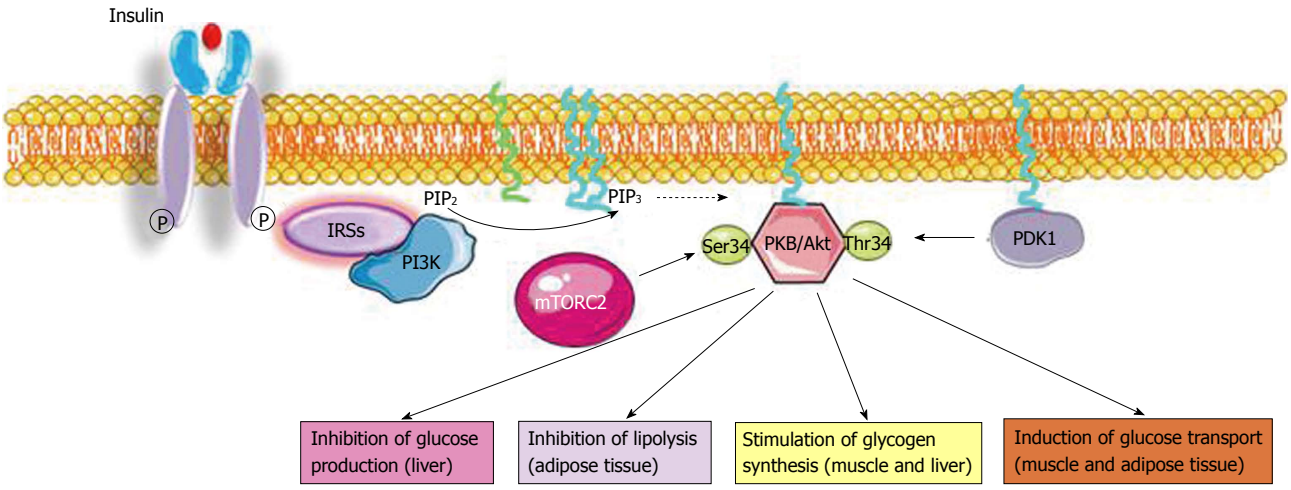

Figure 1 Insulin signaling pathway. Insulin binds with insulin receptor (IR) that activates the IR substrates (IRSs), the phosphoinositide-3-kinase (PI3K), and protein kinase B/Akt (PKBAkt). Activated PKB/Akt mediates insulin metabolic effects and regulates nutrients homeostasis. PIP_2: Phosphorylate phosphatidylinositol, 4, 5-bisphosphate; PDK1: 3-phosphoinositide-dependent protein kinase 1; mTORC2: Mammalian target of rapamycin complex 2.

Ceramide is a bioactive sphingolipid that has been implicated in mediating or regulating many cellular processes, including cell cycle arrest, proliferation, apoptosis, senescence, and stress responses. Ceramide plays also an important role in cell membrane structure[90].

Formation of ceramide can be induced by different stimuli such as tumor necrosis factor-α, heat stress, oxidative stress, ionizing radiation, and chemotherapeutics[91].

Multiple metabolic pathways converge to ceramide (Figure 2): (1) The *de novo* synthesis pathway from saturated fatty acids that takes place in the endoplasmic reticulum; (2) The sphingomyelinase pathway that uses sphingomyelinase to break down sphingomyelin in the cell membrane to release ceramide; and (3) The salvage pathway in lysosomes that occurs through breakdown of complex sphingolipids to give sphingosine, which is then rescued by reacylation to form ceramide.

In time of fatty acid plethora, the *de novo* ceramide biosynthesis pathway is the pathway that is likely to be most harnessed to synthesize ceramide. It occurs in the leaflet membrane of the endoplasmic reticulum where ceramide is synthesized through a series of reactions[92,93]. *De novo* synthesis of ceramide begins with the condensation of palmitate and serine to form 3-keto-dihydrosphingosine (Figure 2). This reaction is catalyzed by serine palmitoyl transferase (SPT) and is the rate-limiting step of the pathway. In turn, 3-keto-dihydrosphingosine is reduced to dihydrosphingosine, which is then followed by acylation by ceramide synthases (CerS) to produce dihydroceramide. In mammals, six CerS isoforms are expressed and are called CerS 1 to 6. They carry out the same chemical reaction, but display distinct specificities for the acyl-CoA chain length they use for N-acylation[94]. Thus, CerS isoforms are responsible for the fatty acid composition of ceramide. Interestingly, several studies have shown distinct cellular functions for ceramides with different N-acyl chain length[95,96]. The final reaction to produce ceramide is catalysed by dihydroceramide desaturase.

Inverse relationship between ceramide content and insulin sensitivity

Studies in animal and models: One of the early studies that analyzed ceramide content in obese Zucker fa/fa rats (rats homozygous for truncated, non-functional leptin receptor) was Turinsky *et al*[97] in 1990. The authors found that these rats present an increase in ceramide content in both muscle and liver. Increased ceramide content was also detected in insulin resistant models of rodents, as in ob/ob mice, mice fed on high fat diet, and in intra-lipid infused mice[85,98,99]. Altogether these reports illustrate the inverse relationship between ceramide and insulin sensitivity in rodent muscle. This association was also confirmed *in vitro* in cultured C2C12 and L6 myotubes, as well as in adipocytes[99-101]. Exposing cultured muscle cells to saturated fatty acids (like palmitate) attenuates insulin activation of glycogen synthesis and glucose transport concomitantly with increasing intracellular ceramide amounts[63,99]. Additionally, incubation of muscle cells and adipocytes with analogues of ceramide mimics the inhibitory effects of FFAs on insulin signaling and suppresses insulin-stimulated glycogen synthesis and glucose transport[100,101].

Studies in human subjects: In accordance with data obtained in rodents, studies in human subjects also support the inverse relationship between ceramide accumulation and insulin sensitivity. It has been shown that under basal conditions, total amount of ceramide in skeletal muscle is increased in obese subjects compared to lean ones[83,84,87]. Another study performed in human skeletal muscle of lean normoglycemic subjects revealed again an inverse relationship between muscle ceramide accumulation and insulin sensitivity[102]. The same authors show in another study a ceramide accumulation in muscle of type 2 diabetic patient offsprings compared to muscle of control subjects[77]. Furthermore, the group of Goodpaster demonstrated that physical exercise reduces ceramide

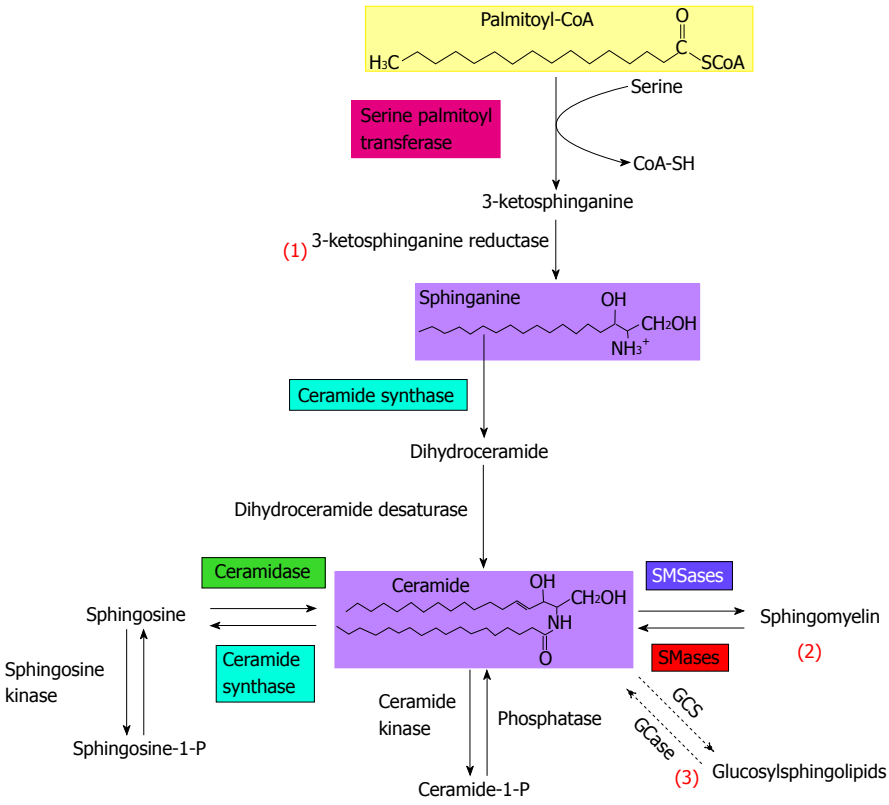

Figure 2 Sphingolipid metabolism. Ceramide can either be newly synthesized in *de novo* ceramide synthesis pathway (1), or it can be the product of complex sphingolipids degradation, including sphingomyelin hydrolysis (2). The degradation of glycosylsphingolipids constitutes the salvage pathway (3). GCase: Glucosyl ceramidase; GCS: Glucosylceramide synthase; SMases: Sphingomyelinases; SMSases: Sphingomyelin synthases.

content in obese and insulin resistant subjects, and this was correlated with improved insulin sensitivity[83,103]. Like in muscle, accumulation of ceramide content in human adipocytes has also been demonstrated to be related to insulin resistance[104,105].

Altogether, these studies prove a solid association between insulin resistance and an increase in ceramide content in both muscle and adipocytes.

Unlike in muscle and adipose cells, a role of ceramide in the onset of hepatic insulin resistance is more debated. Indeed, some studies see no ceramide accumulation in fatty liver[68,70,71], making improbable these lipids as mediators hepatic insulin resistance. This is in contradiction with another study showing increases in hamster hepatic ceramide levels in response to lipopolysaccharide administration[106]. In addition, Longato *et al*[107] saw a dysregulated ceramide metabolism in high fat diet-induced hepatic steatosis.

Interestingly, and in opposite to muscle and adipose tissue, ceramide cannot accumulate in the liver. Indeed, very recently, Watt *et al*[108] have shown that lipid infusion in healthy subjects resulted in a rapid hepatic secretion of ceramide in the circulation, primarily within very low-density lipoprotein[109,110], thereby protecting the liver from the deleterious effects of their intracellular accumulation. It would be interesting, however, to assess whether lipid-induced ceramide secretion is affected in fatty liver (steatosis).

Altogether, if ceramide does not seem to accumulate in liver during lipotoxic conditions, its secretion into the circulation could be deleterious for other peripheral tissues such as pancreatic β cells and muscle cells.

Implication of ceramide in the progression of insulin resistance

Two methods were used to validate the implication of ceramide in impaired insulin sensibility: the first one was to inhibit ceramide production, and the second was to enhance ceramide metabolism towards less harmful sphingolipid species.

Inhibition of ceramide production improves insulin sensitivity: One method used to demonstrate the role of ceramide in the onset of insulin resistance was to inhibit ceramide biosynthesis. The most commonly studied molecular target involved in suppressing ceramide production is the enzyme SPT, enzyme that catalyzes the initial rate-limiting step in *de novo* ceramide synthesis (Figure 3)[90]. Several potent inhibitors of SPT have been documented, although the most widely used is myriocin, a naturally occurring fungal metabolite isolated from Myriococcum albomyces[111]. In studies carried out *in vivo*, administration of myriocin was found to attenuate PKB/Akt inhibition in response to lipid infusion or high-fat feeding, as well as improving glucose tolerance and peripheral insulin sensitivity in obese ob/ob mice and Zucker Diabetic Fatty rats[112-114]. As expected, these beneficial effects of myriocin were associated with reduced

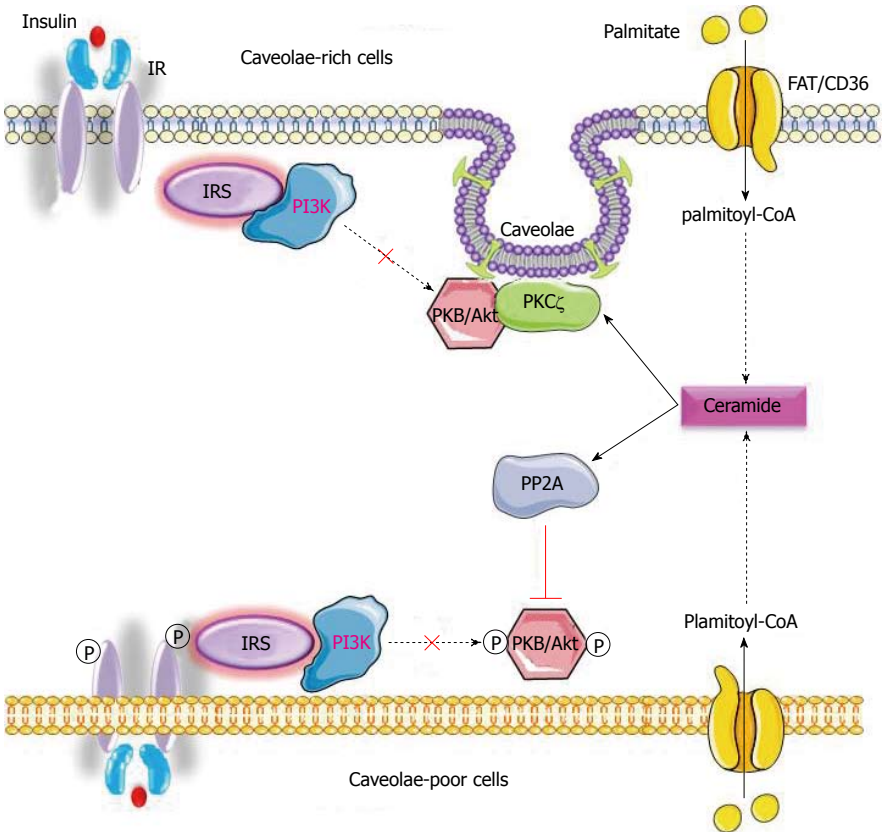

Figure 3 Ceramide inhibits insulin-induced activation of protein kinase B *via* two distinct mechanisms. Ceramide inhibits insulin-activation of protein kinase B (PKB/Akt) either by activating atypical PKC (PKCζ) or by stimulating the phosphatase PP2A. Activation of either mechanism depends on plasma membrane enrichment with caveolae: (1) Ceramide-activated PKCζ phosphorylates the PH domain of PKB/Akt on Thr/Ser34, changing the recognition site of PKB/Akt, and disabling its activation by PIP3; (2) PP2A dephosphorylates PKB/Akt, inhibiting its kinase activity. IR: Insulin receptor; IRS: IR substrates; PI3K: Phosphoinositide-3-kinase; PP2A: Protein phosphatase 2A.

levels of ceramide and were reproduced when alternative inhibitors of *de novo* ceramide synthesis such as L-cycloserine (which also inhibits SPT) and Fenretinide (dihydroceramide synthase inhibitor) were used[63,115].

Studies performed *in vitro* in myotubes confirmed what was observed *in vivo*. They demonstrated that acute inhibition of SPT using myriocin ameliorates the loss in insulin-stimulated PKB/Akt activation in cultured L6 or C2C12 myotubes caused by palmitate-driven ceramide synthesis[62,63].

Interestingly, a very recent study shows that inhibition of the *de novo* synthesis of ceramide using myriocin reduces hepatic lipid accumulation in liver of rats with NAFLD[116]. This inhibition of ceramide biosynthesis is accompanied with decreased in both DAG and triglyceride contents, resulting in amelioration of hepatic insulin resistance and improvement of glucose homeostasis[116].

Stimulation of ceramide conversion into less harmful sphingolipids improves insulin sensibility: The degradation of ceramide is initiated by the action of ceramidase that produces sphingosine, which is then phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase[117]. S1P is the final metabolic product of sphingolipid degradation and can function as an intracellular second messenger or in an autocrine and/or paracrine manner to activate and signal through S1P receptors[118]. Interestingly, S1P itself opposes the effects of ceramide on intracellular signaling. S1P has been shown to ameliorate insulin-stimulated glucose uptake, possibly through the activation of PKB/Akt[118-121]. Therefore, studies have aimed at finding ways to enhance ceramide metabolism into S1P in muscle in order to restore their insulin sensitivity. Bruce *et al*[122] used transgenic mice overexpressing sphingosine kinase. They show that high fat fed transgenic mice display improved insulin sensitivity compared to control mice. In addition, they used a drug called FTY720 which inhibits ceramide synthase activity and decrease ceramide accumulation in skeletal muscle[123]. As expected, they saw an improvement of insulin sensitivity. FTY720 prevented muscle ceramide accumulation in high fat fed mice and subsequently improved glucose homeostasis[124]. Other studies show that overexpression of ceramidase (converting ceramide to sphingosine) protects from lipid-induced muscle insulin resistance in C2C12 myotubes[125].

Altogether, these results demonstrate that preventing the aberrant accumulation of ceramide by promoting its metabolism into sphingosine and sphingosine-derivatives might restore normal insulin sensitivity and glucose me-

tabolism in models of insulin resistance.

Ceramide inhibitory effect on the insulin signaling pathway

Several studies have reported that ceramide may attenuate insulin-stimulated glucose transport and glycogen synthesis by antagonizing early events in insulin signaling such as activation of IRS-1[126] and possibly PI3K[127]. However, these results are controversial, as several groups reported no defects in the activation of these molecules upon challenging cells with ceramide[100,101]. In contrast, a number of groups suggested that PKB/Akt is the target of ceramide, and that inhibition of this kinase may account for reduced glucose transport and apoptosis observed in ceramide treated cells[99-101,128]. Consistent with this, defects in PKB/Akt activation have been noted in a variety of ceramide-treated cell types, including 3T3-L1 adipocytes[101], foetal brown adipocytes[129], L6 rat and C2C12 mouse skeletal muscle[99,100], A75R5 smooth muscle cells[130], and MCF7 breast cancer cells[131].

Furthermore, the inhibition of PKB/Akt by ceramide is not limited to experiments using exogenously supplied lipids. The hormonal activation of PKB/Akt is also blunted in muscle cells treated with free fatty acids in a manner which is dependent on the intracellular conversion of palmitate to ceramide[62,63,99]. Taken together these results suggest that ability of ceramide to impair PKB/Akt activity may be an important determinant of insulin sensitivity.

A key issue is the mechanism by which ceramide inhibits PKB/Akt activity. Depending on the cell enrichment in caveolin-enriched domain[132], ceramide inhibits the insulin-stimulated PKB/Akt either through the protein phosphatase 2A (PP2A), or *via* the atypical PKC (aPKC) pathway (Figure 3).

PP2A depended inhibition of insulin-induced activation of PKB/Akt: PP2A is a cytoplasmic serine/threonine phosphatase ubiquitously expressed that plays an important role in the regulation of diverse cellular processes, including metabolic enzymes, hormone receptors, kinase cascades, and cell growth[133]. It has been shown that insulin inhibits PP2A in physiologic conditions[134]. In contrast, several groups demonstrated that ceramide activates PP2A to promote the de-phosphorylation of PKB/Akt[62,135,136]. Two different inhibitors of PP2A activity, okadaic acid or SV40 small T antigen that binds with PP2A[137] were used to demonstrate the role of ceramide-induced PP2A inactivation of PKB/Akt. The presence of either inhibitor in cells treated with palmitate or short chain ceramide analogue (C2-ceramide), alleviated inhibition on PKB/Akt and re-established a normal, insulin signaling[62,128]. Therefore, one way for ceramide to inhibit PKB/Akt activity is by promoting its dephosphorylation at Thr308 and Ser473 through activation of PP2A.

Atypical PKCs another ceramide-stimulated protein altering PKB/Akt activation: The second mechanism of inactivation of PKB/Akt by ceramide requires the activation of aPKCs (PKCζ/λ). There is mounting evidence in the literature suggesting that aPKC may regulate PKB/Akt signaling and that the relationship between the two kinases may be subject to modulation by ceramide. It is 20 years since investigators first demonstrated that PKCζ/λ could associate with PKB/Akt in COS-7 fibroblasts[138]. It has also been demonstrated that PKCζ interacts directly with PKB/Akt in other cells types such as Chinese hamster ovary cells and COS-1 cells[139], as well as the BT-549 human breast cancer cell line[140].

In pathological conditions, ceramide-activated aPKCs impair insulin signaling. aPKCs phosphorylate PKB/Akt on its Thr34/Ser34 residue (Thr34 in PKBα and PKBβ, Ser34 in PKBγ), thus preventing PIP_3 to bind the kinase on its PH domain, and to translocate to the plasma membrane and its subsequent activation in response to insulin[132,141,142]. Based on these observations, it was proposed that an increase in intracellular ceramide leading to the activation of aPKCs promotes the stabilization of the aPKC-PKB/Akt complex and attenuates the recruitment of PKB/Akt to the plasma membrane as a result of disrupted PIP_3 binding (Figure 3).

CERAMIDE, A THERAPEUTIC TARGET?

Mechanisms by which saturated fatty acids act on insulin signaling are now getting clearer. They involve several lipid and protein intermediates that play an essential role to mediate the deleterious effects of accumulated saturated lipids in insulin sensitive tissues. Thus, two main options exist to counteract the action of these fatty acids on insulin signaling: (1) acting on ceramide downstream signaling targets (aPKCs or PP2A); or (2) modulating directly ceramide content[143]. Considering the large involvement of both aPKCs and PP2A in numerous paths[144,145], it would be more logical to try to directly inhibit the accumulation of ceramides in tissues. Several problems would arise with a complete inhibition of ceramide biosynthesis since these bioactive sphingolipids are in the center of sphingolipid metabolism. Indeed, ceramide signaling has been directly or indirectly involved in the diverse functions such as regulation of cell growth, differentiation, senescence, necrosis, proliferation, and apoptosis[90]. Therefore, inhibiting completely ceramide biosynthesis would be likely to be very harmful to the cells. Targeting specific ceramides species would be more appropriate since it has been shown that specific ceramide species could be associated with different functions, depending upon the cell type[94].

Concretely, it will be important to determine which ceramide species accumulate under lipotoxic conditions and then to evaluate whether these identified ceramide species enhance or reduce the deleterious effects of lipotoxicity in insulin sensitive tissues.

Interestingly, data existing already suggest that ceramide with distinct acyl chain-length are associated with different cell dysfunction in lipotoxic conditions. The

enzyme responsible of generating different ceramide acyl chain-length is the CerS. Six mammalian CerS have been described, with each utilizing fatty acyl CoAs of relatively defined chain lengths for ceramide synthesis[94]. In pancreatic β-cells, C18:0, C22:0 and C24:1 ceramides induce apoptosis, and inhibition of the CerS (CerS4) responsible for their synthesis blocks this phenomenon[146]. In the liver, CerS1 and CerS6, producing mainly C16:0 and C18:0 ceramides are associated with insulin resistance[147], whereas C22:0 and C24:0 ceramides produced through CerS2 are rather protective[148].

In muscle cells, however, no definitive and conclusive investigation has been carried out to date. The expression of C16:0, C18:0 and C24:0 ceramide species are increased in myotubes of type 2 diabetic patients compared to lean donors[149]. However, one recent paper shows that overexpression of each CerS isoform in L6 muscle cells does not point out any ceramide species in the generation of insulin resistance[150]. Since the implication of ceramide in the onset of insulin resistance in muscle has been convincingly demonstrated both *in vivo* and *in vitro* (see previous chapters), more investigations are needed before to make any conclusion in this tissue.

In summary, deciphering the mechanisms by which ceramides act negatively on insulin signaling has already been a step forward. However, the identification of the putative ceramide species that mediates lipotoxicity in cells or pushing ceramides to be converted into less toxic lipids remains the priority in order to find a way to counteract ceramide negative actions.

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P- Reviewer: Sasaoka T **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Adrenomedullin and diabetes

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Supported by The University of Hong Kong Seed Funding Program for Basic Research, No. 201111159132; and the Sun Chieh Yeh Heart Foundation
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Received: September 25, 2013 Revised: April 10, 2014
Accepted: May 8, 2014
Published online: June 15, 2014

Abstract

Adrenomedullin (ADM) is a peptide hormone widely expressed in different tissues, especially in the vasculature. Apart from its vasodilatatory and hypotensive effect, it plays multiple roles in the regulation of hormonal secretion, glucose metabolism and inflammatory response. ADM regulates insulin balance and may participate in the development of diabetes. The plasma level of ADM is increased in people with diabetes, while in healthy individuals the plasma ADM concentration remains low. Plasma ADM levels are further increased in patients with diabetic complications. In type 1 diabetes, plasma ADM level is correlated with renal failure and retinopathy, while in type 2 diabetes its level is linked with a wider range of complications. The elevation of ADM level in diabetes may be due to hyperinsulinemia, oxidative stress and endothelial injury. At the same time, a rise in plasma ADM level can trigger the onset of diabetes. Strategies to reduce ADM level should be explored so as to reduce diabetic complications.

Key words: Adrenomedullin; Diabetes; Diabetic complications; Hyperglycemia; Therapeutics

Core tip: Adrenomedullin (ADM) is a peptide hormone with vasorelaxing and hypotensive properties. It also plays multiple roles in the regulation of hormonal secretion, glucose metabolism and inflammatory response. A major observation is the elevation of plasma ADM level in diabetes, and is associated with diabetic complications in both type 1 and 2 diabetes. The increase could be resulted from oxidative stress, hyperinsulinemia and endothelial injury. This raises the potential application of ADM as a marker in diabetes, and strategies aimed at reducing ADM level could be explored so as to alleviate diabetic complications.

Original sources: Wong HK, Tang F, Cheung TT, Cheung BMY. Adrenomedullin and diabetes. *World J Diabetes* 2014; 5(3): 364-371 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/364.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.364

INTRODUCTION

Adrenomedullin (ADM) is a peptide recently discovered with multiple functions. Its characteristic actions include vasorelaxing effect and hypotensive properties. Given its widespread expression and production in different organs, ADM can also act as an autocrine, endocrine or paracrine mediator in various biological systems. The prospects of ADM as a potential disease modulator comes from the observation of increased levels in plasma in various disease states. For instance, increased plasma ADM levels were observed in cardiovascular diseases and diabetes[1-3]. However, different from the observations in cardiovascular diseases, the explanation and significance for such an increase is not clear. Since then, research progress has been made in the association between ADM

and diabetes. For instance, ADM plays a role in glucose metabolism and insulin balance[4]. These evidence may provide clue on the involvement of ADM in diabetes.

In this review, we summarized the current knowledge on ADM based on research progress in the recent decade and provided an account on the role of ADM played in the context of diabetes. This would help us understand better on the clinical application of ADM in diabetic patients.

DISCOVERY OF ADRENOMEDULLIN AS A REGULATORY PEPTIDE

ADM was initially discovered by Kitamura in 1993, extracted from pheochromocytoma in humans by monitoring the elevated 3',5' cyclic adenosine monophosphate (cAMP) production in human platelets[5]. It was later found that the peptide had a potent hypotensive and vasorelaxing effects. It forms a ring structure by 52 amino acid residues held by a disulfide bond. Since the peptide was abundantly found in the adrenal medulla, therefore this accounts for the name. The peptide is classified as a member of the calcitonin gene-related peptide (CGRP) superfamily. Although high level of ADM was identified in the adrenal medulla[6], circulating ADM was the most abundant in vascular wall[7].

BIOSYNTHESIS AND DISTRIBUTION

ADM has a very high tissue distribution. Its biosynthesis has been studied by applying radioimmunoassays, and by detecting tissue ADM mRNA[8]. Immunoreactive ADM is detected in cardiovascular, respiratory, renal, endocrine, reproductive, neurological, intestinal and immune system[9,10]. Among these systems the highest ADM concentrations were detected at the adrenal glands. ADM mRNA is also detected in various peripheral tissues[11]. Such wide distributions indicate the multi-facet roles of ADM.

In the cardiovascular system, ADM is synthesized in both atria and ventricles in heart and blood vessels. Within the vasculature, ADM is actively manufactured and secreted by both the endothelial and the vascular smooth muscle cells[7,12]. It is also demonstrated that the vasculature had much higher ADM mRNA expression than the adrenal glands. This was further supported by the finding of a low ADM precursor ratio in the total ADM immunoreactivity in blood vessels[11].

Besides, ADM is synthesized in the lung[13], brain as well as in the pancreatic islets[14,15]. The widespread ADM expression suggests its diverse role in the regulations of cell functions. Since ADM is mainly produced by vascular endothelial and the smooth muscle cells, its regulatory function of vascular tone has become a major target for investigation.

ADM production is controlled by various humoral factors and physical factors. Inflammatory cytokines such as tumor necrosis factor (TNF)-α, TNF-β, interleukin (IL)-1α and IL-1β all are known to stimulate ADM production and secretion[16]. While mechanical factors like sheer stress and hypoxia are involved in the up-regulation of vascular ADM mRNA expression[17].

In healthy individuals, circulating plasma ADM level is as low as in the picomolar range, similar to the atrial natriuretic peptide, and its level changes in order to compensate for the vasoconstrictive effects. It is reported that in various pathological conditions, the increase in plasma ADM level correlates with severity of disease states. For instance, elevated plasma ADM level has been associated with heart failure, hypertension, artherosclerosis and diabetes mellitus[18].

RECEPTOR SIGNALING

Specific binding sites for ADM were identified in many different places in rat and in human models[19,20]. In humans, the binding sites are most abundant in the microvascular endothelium[20]. The biological actions of ADM are exerted mainly through CGRP receptors and the specific ADM receptors, which share a common molecular component of a G-protein coupled receptor called calcitonin receptor-like receptor (CRLR)[21]. The specificity of CRLR depends on different subtypes of another associated proteins, namely the receptor-activity-modifying proteins (RAMP1, 2 and 3)[22]. Co-expression of CRLR with different subtypes of RAMPs will form different ADM receptors. The specificity brought about by the RAMPs involves glycosylation and transport of the receptor-RAMP complex.

PHYSIOLOGICAL EFFECTS

ADM can act as both a hormone and a cytokine to regulate the regional blood flow, vascular tone, leukocyte migration and differentiation, electrolyte balance, cardiac function, glucose uptake and hormone secretion[18]. It plays an important role in cardiovascular system[23]. ADM imposes a potent vasodilatory effect in humans and increases blood flow to various organs[24,25]. For instance, increased ADM expression could enhance hepatic and renal circulation[26]. In systemic circulation, vasodilation could be resulted from either endothelium-dependent[27], or endothelium-independent mechanisms[28], through ADM and CGRP receptors. In addition, the endothelium-derived vasodilation could be mediated by cAMP and nitric oxide[29,30].

Previous studies have identified the role of ADM in inflammation and immunity. ADM possesses anti-microbial properties against bacteria[31]. *In vitro* and *in vivo* study has demonstrated that ADM secretion and expression are up-regulated upon pathogenic exposure[32]. ADM expression also increases during local inflammation and sepsis[33] In particular, ADM levels in lung, heart and vasculature[34], liver and kidney[26], all increase upon endotoxin administration[35]. Macrophages could also augment ADM expression in inflammation[33].

The role of ADM in the inflammatory process var-

ies after the onset of inflammation. ADM can activate and modulate cytokine production, while it can also inhibit overproduction of pro-inflammatory cytokines[36]. It plays a crucial role in initiating inflammatory response by stimulating the release of migratory inhibitory factor and IL-1β, while activate anti-inflammatory response by suppressing TNF-α production and up-regulating IL-6 production, as the latter is anti-inflammatory and inhibit lipopolysaccharide-induced TNF-α production[37-39]. Such co-ordinated functions of ADM suggest that it is associated with injury, infection and inflammation. Apart from inflammation, ADM expression in immune cells serves diverse functions. ADM can be detected in macrophages in the atherosclerotic plaques[40], where it may play a role in reducing inflammation and thereby exerting an anti-atherosclerotic effect.

While circulating ADM in plasma contributes to a large part of its physiological functions, ADM also serves as a local regulator of cellular functions. The paracrine effect of ADM can be demonstrated in the kidney, as it has been shown that ADM is histochemically localized in renal tubules, and recently mesangium was suggested to be one source of ADM in the kidney[41]. The local ADM modulates mesangial proliferation and is regulated by different growth factors and cytokines. This suggests that regulation of renal function by ADM may operate in an autocrine/paracrine manner. Another example of the localized effect of ADM is in the vascular smooth muscle cells, where its biosynthesis is regulated through a feedback loop. In one study, stimulation of ADM mRNA levels was observed together with a decrease in the immunoreactive ADM peptide secretion resulted from glycolytic inhibition[42]. As ADM could inhibit vascular smooth muscle cell migration and proliferation in response to growth factors[43], a decreased ADM secretion might stimulate its migration and growth locally, and lead to remodeling upon vascular injuries.

ADRENOMEDULLIN AND PANCREATOLOGY

ADM is deeply involved in pancreatic endocrinology, mainly in insulin secretion[44]. It is known that ADM, CRLR and RAMPs are both expressed in the islets of the pancreas[45]. Previous findings demonstrated that exogenous ADM added to freshly isolated rat islets led to a dose-dependent inhibition of insulin secretion by 78% at 1 μmol/L ADM, and was accompanied by cAMP elevation[3]. Oral glucose tolerance tests have illustrated injection of ADM lowered insulin levels in blood by 2 folds 20 min after glucose administration, accompanied by an increase in circulating glucose[4]. This supports a role of ADM in insulin regulation in pancreas, and implies that ADM is associated with hyperglycemia[46].

Another function of ADM is inhibiting amylase secretion in pancreatic acini[47]. As ADM receptors were not identified in the acini, this suggest that such inhibition is mediated through other receptors[45].

ADRENOMEDULLIN AND DIABETES

As suggested above, ADM inhibits insulin release after an oral glucose load. Therefore, it can be expected that ADM contributes to diabetes and even leads to the development of diabetic complications[48].

Diabetes is characterized by hyperglycemia. It is resulted from dysregulation of insulin secretion or peripherial resistance. Diabetes mellitus causes retinopathy, neuropathy, nephropathy, and atherosclerosis. These complications are the results of prolonged hyperglycemia, altered metabolic pathways and non-enzymatic glycation of proteins[49].

There have been advances in the understanding of the relationship between ADM and diabetes. Plasma ADM level is elevated in patients with poorly controlled diabetes than in normal subjects, which suggests a direct effect of glucose on ADM release[1]. The effect of hyperglycemia on ADM expression is mediated through protein kinase C in vascular smooth muscle cells[50]. The observation that ADM expression in aorta, but not in adrenal gland, was raised in diabetic rats (plasma glucose = 567 ± 167 mg/dL) compared to control (plasma glucose = 94 ± 10 mg/dL), suggests that ADM expression in the vasculature could be the source of plasma ADM in diabetic patients[50]. In the streptozotocin-diabetic rat, there were increases in ADM synthesis in the ventricles and possible ADM secretion in the ventricles, atria and the thoracic aorta[51]. On the other hand, ADM may reduce the levels of inflammatory cytokines and endothelin in the adipose tissue and the skeletal muscle and hence increase glucose uptake[37].

However, another study examining the relationship between plasma ADM level and clinical parameters of diabetes demonstrated contradictory results. It showed no significant difference in plasma ADM level between diabetic patients without nephropathy and normal individuals, despite a significant higher level of HbA1c and plasma glucose in patients with diabetes[52]. Therefore, patients with renal impairment should be excluded when examining the relationship between plasma ADM level and blood glucose level, since patients with renal impairment might demonstrate an increase in the plasma ADM levels. Despite the direct effect of circulating glucose on plasma ADM level has not been well established, a positive association between plasma ADM level and the mean blood pressure has been demonstrated in the same study. Given the high plasma ADM levels in various disorders[53], the elevated ADM levels in diabetes might suggest that it has a protective role. Earlier research also showed an elevated plasma ADM level in patients with hypertension and chronic renal failure, particularly a 3-fold elevation in plasma ADM level associated with more severe renal failure. The elevation in ADM may help to prevent blood pressure increase and body fluid retention[54], and represent a compensatory mechanism for diabetic

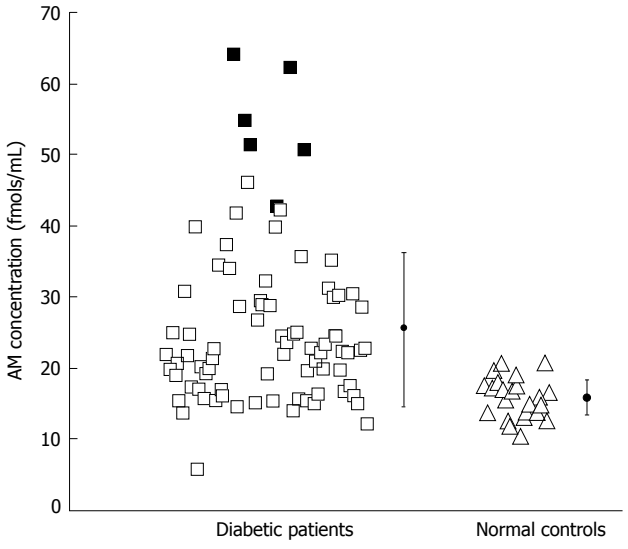

Figure 1 Adrenomedullin concentrations in blood serum from type 2 diabetic patients (in squares) and normal controls (in triangles), shaded squares are outliers. Reprinted from [63].

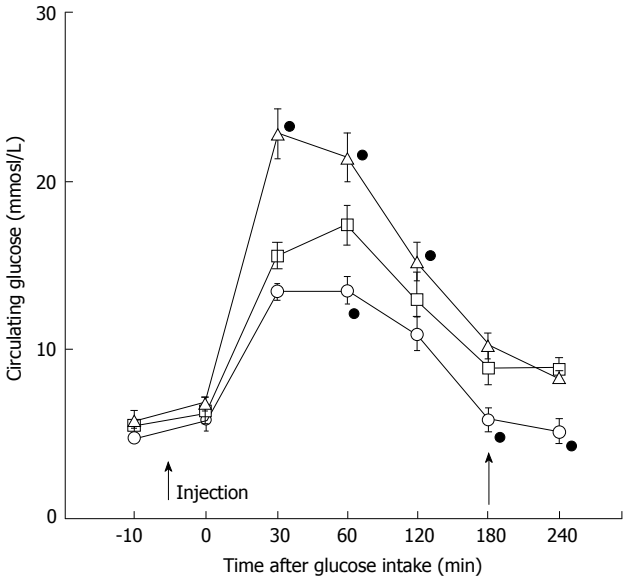

Figure 2 Glucose tolerance test in obese diabetic SHR/N-cp rats after injection of saline (squares), adrenomedullin (triangles) and anti-adrenomedullin monoclonal antibody MoAb-G6 (circles). Black spots indicates point with significant difference compared with saline controls. Modified and reprinted from [63].

complications.

ADM AND TYPE 1 DIABETES

One characteristic of type 1 diabetes is the destruction of β-cells in the islets of Langerhans which produces insulin. Previously there was a report investigating the association of ADM and type 1 diabetes. ADM and cAMP levels were compared between type 1 diabetes patients with various complications and healthy individuals[55]. According to the data, increased plasma ADM level was identified only in patients having renal insufficiency, while patients with other complications had normal ADM level. A significant inverse correlation was also found between ADM levels and the creatinine clearance by multiple regression analysis. This suggested that when the kidney function was impaired, clearance of ADM was possibly decreased and resulted in an increase in the plasma level. Such hypothesis deserves further confirmation because most of the circulating ADM was shown to be cleared in the lungs instead of the kidneys[56]. In the same analysis, the relationship between the plasma ADM and the disease duration suggested the change in ADM level is resulted from the endothelial dysfunction.

Despite the uncertainty of the origin of plasma ADM, a recent study postulated that the selective dilation of glomerular capillaries in type 1 diabetes was attributed to the up-regulation of ADM and RAMP2 expression in the afferent arterioles and glomeruli, through the induced release of nitric oxide[57]. This may provide a hint that locally produced ADM can elicit vasodilatation action by paracrine control, independent of any changes in plasma ADM levels. ADM is also involved in the pathogenesis of retinopathy[58]. Since ADM is produced in the vasculature, endothelial activation caused by vessel damage may explain the increase in plasma ADM level. Another possibility is that ADM acts as a factor for survival of the endothelial cells[59], so plasma level of ADM increases upon endothelial injury. A significant positive association between ADM and cAMP in diabetic patients further supported the hypothesis that ADM plays a counter-regulatory role to prevent excessive vasoconstriction and vessel damage, and promotes natriuresis[54,60,61].

All these findings suggested an increase in plasma ADM level is the consequence rather than the cause of type 1 diabetes, since there are insufficient findings to demonstrate the direct link between ADM and the disease states. This can be further supported by the comparison of hypoglycemic- and hyperglycemic-patients in the same study in which no difference in the plasma ADM level was found.

ADM AND TYPE 2 DIABETES

Several studies have been carried out in an attempt to explain the rise in plasma ADM level and its implications in diabetic complications. One study showed that plasma ADM level was elevated in type 2 diabetes but did not correlate with glucose level in circulation[62]. Instead, increased ADM level was correlated with various diabetic complications, and the severity of diabetic nephropathy and retinopathy. Other parameters like serum creatinine level, systolic blood pressure, and urinary protein excretion were found to be related to ADM levels as well. ADM levels might therefore be related to the development of microangiopathy.

Another study examined a group of patients with a common feature of hyperglycemia development. The group had recent onset of diabetes induced by a drug treatment[63]. Results showed that the group can be characterized by a subset of patients with extremely high

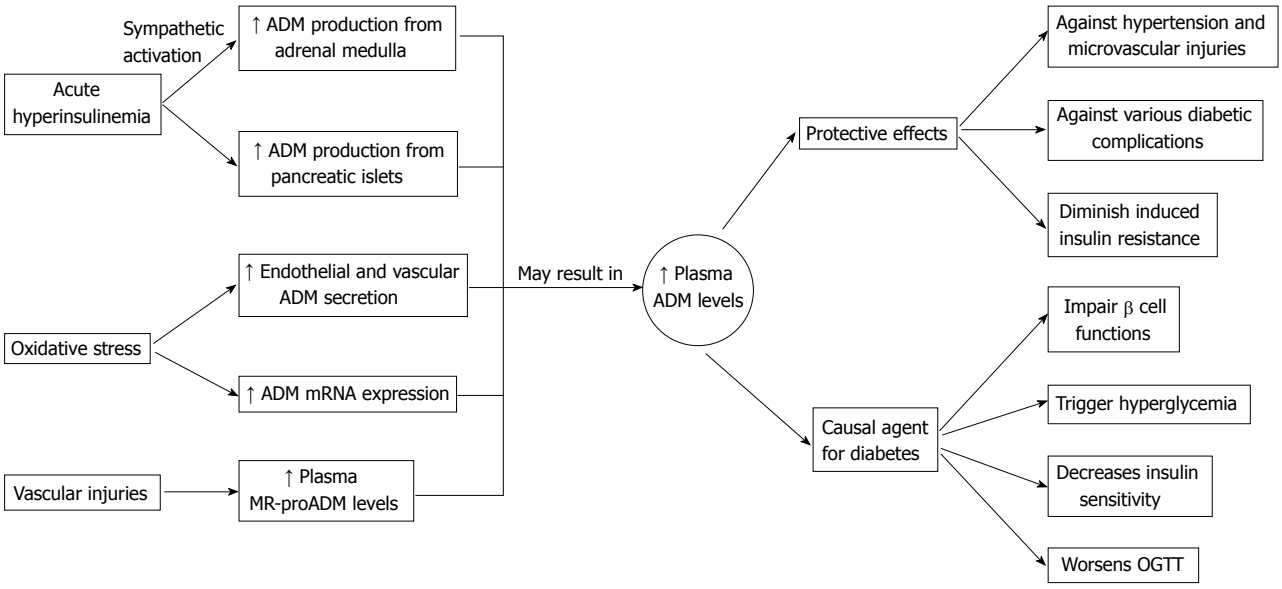

Figure 3 Effects of different disease stresses on subsequent adrenomedullin production and plasma adrenomedullin levels, and the possible roles of increased adrenomedullin levels in type 2 diabetes. ADM: Adrenomedullin.

ADM levels (Figure 1). Even though the source of such excessive ADM is unknown, the results suggested that hyperglycemic patients are characterized by higher circulating ADM levels. In the same studies, the influence of ADM in blood glucose modulation was studied using an obese SHR rat model mimicking human type 2 diabetes. Synthetic ADM, blocking monoclonal antibody against ADM or saline were injected into the animals, and then glucose tolerance tests were carried out. In support to a previous study[4], ADM injection increased blood glucose level more significantly in diabetic rats, while application of antibody effectively reduced blood glucose level to even lower than saline control and improved postprandial recovery in diabetic rats (Figure 2). All these data raise the possibility that ADM is a causative factor in type 2 diabetes and has a negative impact on glycemic control.

To further explore the role of ADM incausing type 2 diabetes, the effect of ADM on insulin secretion has to be considered. There are studies addressing the association of ADM with insulin balance. There is a positive association between insulin resistance and plasma midregion pro-adrenomedullin levels[64]. The link between acute hyperinsulinemia and ADM has been proposed, in which plasma ADM levels increased in acute hyperinsulinemia[65]. There was a concomitant increase in plasma ADM levels with increasing insulin production, and a significant positive correlation between serum insulin levels and plasma ADM was seen in type 2 diabetic patients. The authors speculated that the increased insulin-stimulated ADM production from the pancreatic islets compensated for the diminished vasodilatory effect of insulin, hence this protects against arterial hypertension.

In the recent decade the effect of oxidative stress on ADM expression has been suggested. One study evaluated such relationship by measuring plasma levels of 8-epi-prostaglandin F2α (8-epi-PGF2α, a marker of oxidative stress) and ADM in normal and hypertensive subjects[66]. Both plasma levels were elevated in the hypertensive group ($P < 0.05$ for 8-epi-PGF2α and $P < 0.02$ for ADM respectively), and the data showed that 8-epi-PGF2α was associated with ADM in hypertensive patients with type 2 diabetes ($r = 0.696$, $P < 0.01$). It is known that oxidative stress could stimulate ADM mRNA expression and secretion from endothelial and vascular smooth muscle cells[67]. Sustained ADM deficiency increased oxidative stress and led to insulin resistance *via* impaired insulin signaling, which is supported by an angiotensin (Ang)-Ⅱ treated mouse model[68]. Ang-Ⅱ could induce oxidative stress and hypertensive conditions, and it was shown that Ang-Ⅱ reduced insulin sensitivity in ADM-knockout heterozygous mice more than wild type mice. This suggests that endogenous ADM may act against insulin resistance induced by oxidative stress and offer protection from organ damage through its anti-oxidant action.

The interactions between ADM and diabetic complications are dynamic and complex. While conflicting arguments have been put forward to the link between poor metabolic control and increased ADM levels[64], it is generally accepted that plasma ADM levels are positively linked to oxidative stress[66], acute hyperinsulinemia[65], and other risk factors causing endothelial injury (Figure 3). This leaves much ground for further research about the causes and significance for the plasma ADM level increase.

CONCLUSION

There are two main questions that have to be answered in order to establish a link between ADM and diabetes: Firstly, what are the causes for the increase in plasma ADM levels in diabetic patients, and what are the sources for the elevated circulating ADM? What kind of

stress or stimulation are involved? Secondly, what is the implication for the elevated level? Would it further worsen the glycemic condition and result in various diabetic complications?

Based on the above questions, numerous studies have been commenced. Research has demonstrated the association between diabetic complications and the increase in plasma ADM level. Plasma ADM levels were mainly associated with renal failure and retinopathy in type 1 diabetes. However, the correlation with hyperglycemia is still not clear and requires further investigation.

On the other hand, plasma ADM levels in type 2 diabetes patients are linked to a wider range of complications. The rise may be attributed to acute hyperinsulinemia, oxidative stress and endothelial damage. These stimuli increases ADM production from pancreatic islets and vascular endothelium. Such a rise may represent a causative factor triggering the onset of disease and insulin resistance. If this assumption holds, a controlled reduction in ADM levels may improve hyperglycemia. To understand the casual role of ADM in diabetes, genetic variants could be a potential variable to study using Mendalian randomization, since it is unlikely to be confounded by environmental factors. Our recent study has demonstrated a positive link between a single nucleotide polymorphism (SNP) of ADM gene and development of dysglycemia[69]. Our other studies also demonstrates that plasma ADM level is associated with one of its SNP, IL-6 and adiponectin SNPs[70-72]. In the future regulation of ADM level could be a key in controlling glycemia in people with diabetes and this warrants further investigation.

ACKNOWLEDGMENTS

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P- Reviewers: Romani A, Swierczynski JT, Yin DP
S- Editor: Song XX **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Knockout mouse models of insulin signaling: Relevance past and future

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Received: October 16, 2013 Revised: February 11, 2014
Accepted: Feburary 20, 2014
Published online: April 15, 2014

Abstract

Insulin resistance is a hallmark of type 2 diabetes. In an effort to understand and treat this condition, researchers have used genetic manipulation of mice to uncover insulin signaling pathways and determine the effects of their perturbation. After decades of research, much has been learned, but the pathophysiology of insulin resistance in human diabetes remains controversial, and treating insulin resistance remains a challenge. This review will discuss limitations of mouse models lacking select insulin signaling molecule genes. In the most influential mouse models, glucose metabolism differs from that of humans at the cellular, organ, and whole-organism levels, and these differences limit the relevance and benefit of the mouse models both in terms of mechanistic investigations and therapeutic development. These differences are due partly to immutable differences in mouse and human biology, and partly to the failure of genetic modifications to produce an accurate model of human diabetes. Several factors often limit the mechanistic insights gained from experimental mice to the particular species and strain, including: developmental effects, unexpected metabolic adjustments, genetic background effects, and technical issues. We conclude that the limitations and weaknesses of genetically modified mouse models of insulin resistance underscore the need for redirection of research efforts toward methods that are more directly relevant to human physiology.

Key words: Insulin resistance; Mice; Knockout; Disease models, Animal; Glucose/metabolism; Signal transduction

Core tip: Insulin resistance is central to the pathophysiology of type 2 diabetes. The molecular origins of insulin resistance have been investigated using genetically modified mice. Much has been learned from this work, but new treatments for insulin resistance have not been forthcoming. Knockout mouse models of diabetes are limited by several factors including species differences in glucose metabolism. These are due partly to species differences in physiology, and partly to the failure of genetic modifications to produce an accurate model. Advancement may require a redirection of research efforts toward methods that are more directly relevant to human physiology.

Original sources: Bunner AE, Chandrasekera PC, Barnard ND. Knockout mouse models of insulin signaling: Relevance past and future. *World J Diabetes* 2014; 5(2): 146-159 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/146.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.146

INTRODUCTION

Type 2 diabetes is a growing public health problem affecting approximately 26 million adults in the United States, with pre-diabetes affecting an additional 79 million[1]. The natural history of type 2 diabetes starts with insulin resistance, which develops over time and often precedes a diagnosis by many years. The pancreas com-

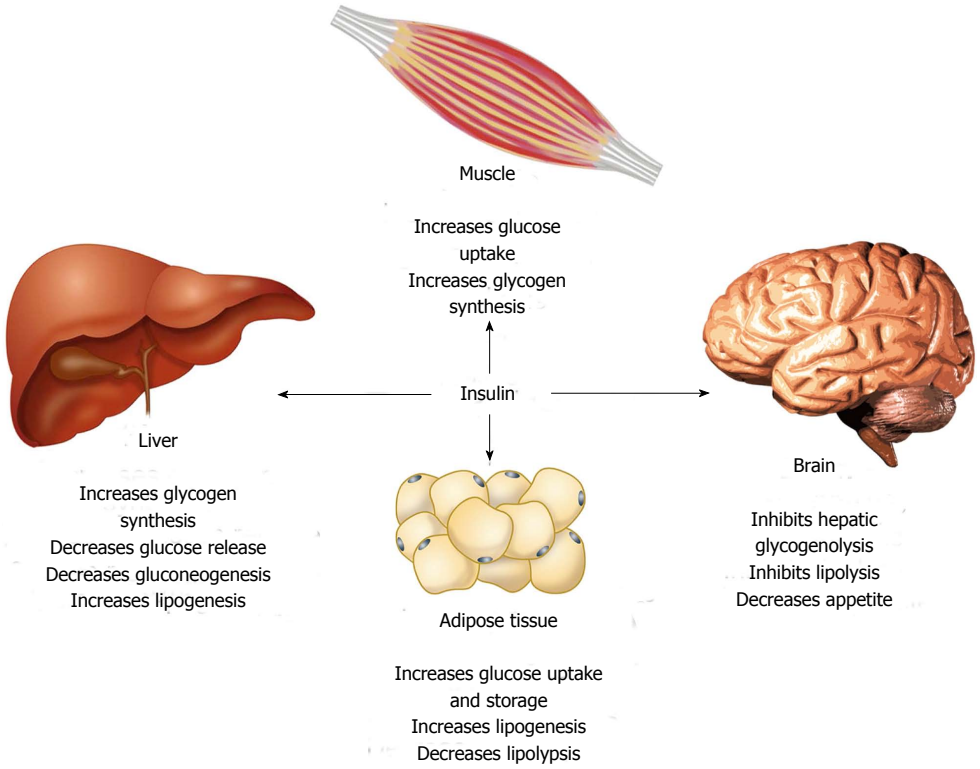

Figure 1 Insulin actions in main insulin-sensitive tissues. Insulin has different actions in each of the main insulin-sensitive tissues. In muscle, insulin promotes glucose uptake and glycogen synthesis. In liver, insulin promotes glycogen synthesis and lipogenesis and reduces gluconeogenesis and the release of stored glucose. In adipose tissue, insulin increases glucose uptake and lipogenesis and decreases lipolysis. In the brain, insulin Inhibits hepatic glycogenolysis and lipolysis and decreases appetite.

pensates for insulin resistance by increasing insulin secretion, often leading to hyperinsulinemia. For many insulin-resistant patients, the pancreas is unable to sustain a high level of insulin secretion. As the pancreas fails to meet the demand for insulin, plasma glucose rises. Patients are then at risk of morbidity and mortality associated with complications such as neuropathy, retinopathy, nephropathy, and increased risk of cardiovascular disease. Overall, type 2 diabetes decreases life expectancy at age 50 or older by about 8 years[2]. Aside from diabetes and the metabolic syndrome, insulin resistance is also associated with polycystic ovarian syndrome and other problems. Understanding the cellular and molecular causes of insulin resistance is an area of active research because of the need to discover new therapies to help patients.

Animal models are often used to investigate mechanisms of insulin resistance and develop therapeutic agents. In the field of type 1 diabetes, serious limitations of animal models have become apparent[3]; we therefore sought to assess the utility of select mouse models used in type 2 diabetes research, specifically insulin signaling and resistance. We begin with a brief summary of insulin signaling, followed by a closer look at general limitations of mouse models and specific limitations of knockouts lacking select insulin signaling molecule genes.

Insulin resistance is defined as the failure of cells to respond normally to insulin, and most importantly, to insulin's glucose-lowering effects. It can be measured by a number of approaches, including the Homeostatic Model Assessment of Insulin Resistance, which is based on fasting glucose and insulin levels, and the gold standard approach, a hyperinsulinemic-euglycemic clamp test[4]. On a cellular level, insulin resistance manifests differently in different tissues (Figure 1). Insulin-resistant muscle cells fail to uptake glucose and other nutrients in response to insulin, whereas in adipose tissue, insulin resistance leads to greater hydrolysis of stored triglycerides in addition to decreased nutrient uptake. In the liver, insulin promotes glycogen synthesis and prevents the release of stored glucose, thereby raising blood glucose levels. In the brain, insulin decreases appetite and hepatic glucose production[5].

The molecular mechanisms of insulin resistance in type 2 diabetes have not been fully characterized, although many important biochemical, metabolic, and genetic features have been identified. Accumulated findings have highlighted several pathways to insulin resistance, including lipid accumulation, oxidative stress, and inflammation[6]. An important common feature of these mechanisms is the activation of stress-sensitive kinases including protein kinase C ζ (PKCζ) that cause a dampening of insulin signaling[6,7].

Insulin is involved in a number of cellular processes apart from nutrient metabolism, including protein synthesis, mitochondrial biogenesis, growth, autophagy, proliferation, differentiation, and migration[8-10]. As illustrated in Figure 2, the binding of insulin to its receptor triggers a cascade of cellular events that leads to nutrient uptake and activation of these various cellular programs[8]. Un-

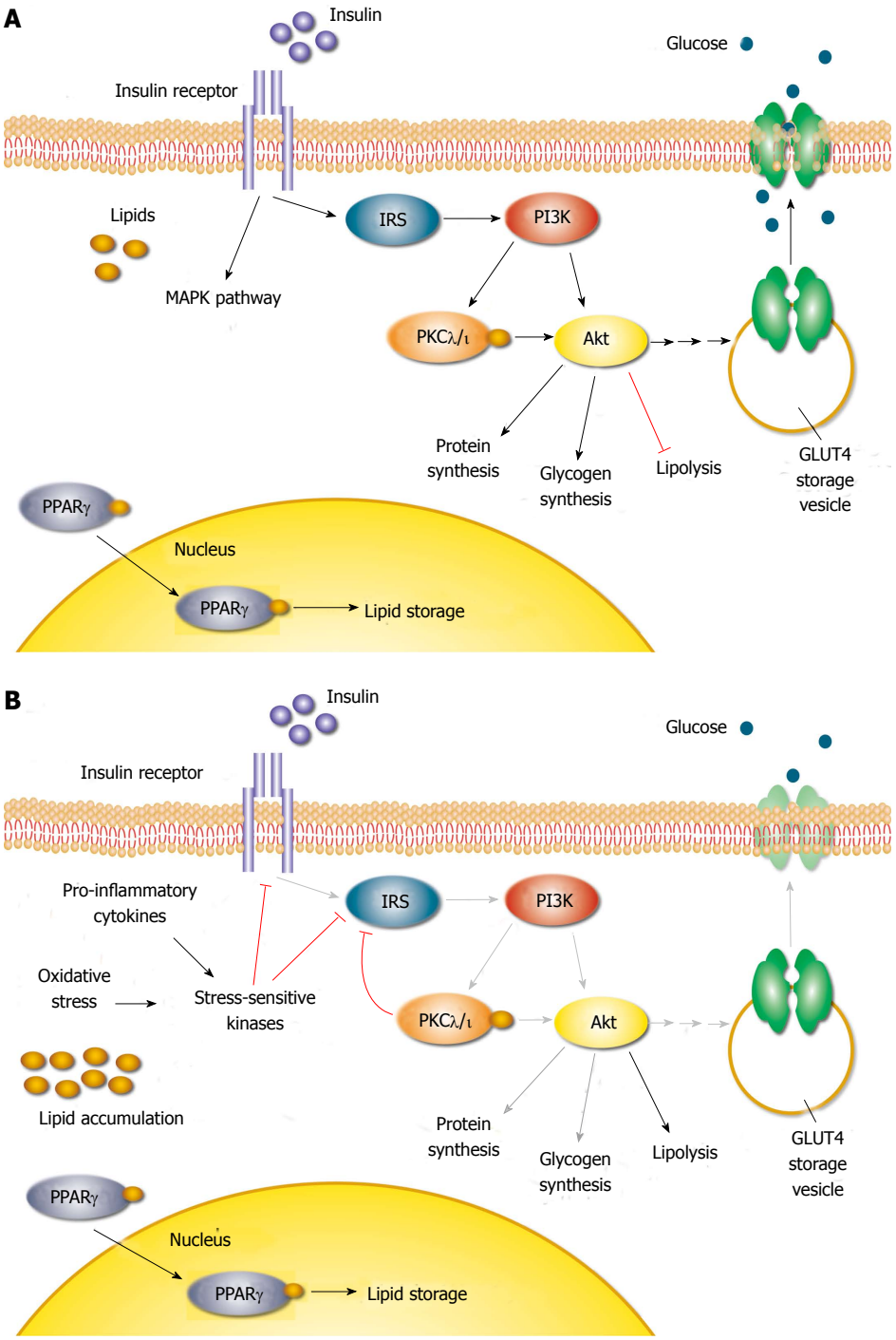

Figure 2 Insulin signaling in health and disease. Insulin signaling in health and disease. A: The binding of insulin to its receptor triggers a cascade of cellular events that lead to nutrient uptake and activation of various cellular programs. Insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K) which produces a metabolite that activates protein kinase B (AKT) and protein kinase C λ/ι (PKCλ/ι). PKCλ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs. PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage; B: Under insulin-resistant conditions, accumulation of lipids, oxidative stress, and pro-inflammatory cytokines cause activation of stress-sensitive kinases such as protein kinase C θ (PKCθ), inhibitor of nuclear factor kappa-B kinase subunit β (IKKβ) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling.

der insulin-sensitive conditions, as shown in Figure 2A, insulin receptor substrate (IRS) activates phosphoinositide 3-kinase (PI3K), which produces a metabolite that activates protein kinase B (AKT) and PKCλ/ι. PKC λ/ι, which also depends on lipids for activation, can inhibit insulin signaling by a feedback mechanism. The nuclear receptor peroxisome proliferator-activated receptor gamma, or peroxisome proliferator-activated receptor γ (PPARγ), is important in lipid metabolism, and is the target of insulin sensitizing thiazolidinedione drugs (TZDs). PPARγ becomes activated upon binding of lipids and promotes expression of genes involved in fat storage. As shown in Figure 2B, under insulin-resistant conditions, accumulation of lipids, oxidative stress, and pro-inflam-

matory cytokines cause activation of stress-sensitive kinases such as PKCθ, inhibitor of nuclear factor kappa-B kinase subunit β (IKK-β) and c-Jun N-terminal kinase 1 (JNK1), which inhibit insulin signaling[6,7].

Evidence for insulin signaling pathways and mechanisms of insulin resistance comes from human and animal cell and tissue studies, clinical studies, and whole animal experiments. While data from various models have been useful in formulating and testing hypotheses, some approaches are more promising than others. Rodent models have been used in the study of type 2 diabetes and insulin resistance for decades. Conditions relevant to the study of insulin resistance and diabetes are induced in rodents using several approaches, including genetic, pharmacological, surgical, and dietary inductions. A number of these approaches and models have been reviewed elsewhere[11-14]. Many researchers favor targeted genetic manipulation because it allows specific and complete or near-complete removal of target gene function in a whole organism or specific tissues[15]. In combination with pharmacological, cell-based and molecular studies, these knockout mouse studies have mapped the insulin signaling pathway in mice to a high level of detail. Other authors have described how pathway connections tested in humans have been shown to be conserved (*i.e.*,[16]). Many would argue that knockout mouse studies have been especially important in defining the function of genes for which no pharmacological or other molecular-based functional ablation is available[17]. In this respect, the genetic approach has become a central component of preclinical research in diabetes and other fields.

Despite this progress in our understanding of insulin action, the causative molecular basis for acquired human insulin resistance remains unclear and controversial. Furthermore, improved understanding of rodent cell signaling has not translated into improved human therapeutics. To wit, it has been almost 20 years since the first insulin signaling knockout mouse studies were published[18,19], but no new drugs targeting the insulin signaling phosphorylation cascade have emerged to treat insulin resistance in type 2 diabetes[9]. While much of this research is conducted for the purpose of hypothesis testing rather than drug development *per se*, the identification of drug targets is often a primary or secondary goal[20]. In light of this, we discuss the limitations of research on insulin resistance using knockout mice of select proteins important in the insulin signaling cascade (Figure 2). The following sections will focus mainly on peripheral insulin resistance and extrapancreatic insulin-sensitive tissues, since many therapeutic and research efforts are in this area. We first address physiological, cellular, and molecular differences in glucose metabolism between mice and humans that limit translatability. We then review select knockout mouse models of insulin signaling dysfunction, identifying cases with contradictory or untranslatable results. Finally, we briefly discuss the limitations of genetic manipulations of these targets in mice in regard to the search for safe and effective drugs for type 2 diabetes.

GLUCOSE DISPOSAL IN MICE AND HUMANS

A central aspect of glucose homeostasis is glucose disposal, meaning the facilitated transport of glucose from blood into storage tissues and organs. Insulin resistance in humans with type 2 diabetes involves defects in glucose sensing and disposal in a number of tissues, but the most significant effects on glucose homeostasis result from insulin resistance in the major glucose-disposing tissues: skeletal muscle, liver and adipose tissue.

Glucose disposal and glycogen storage patterns differ in mice and humans. In healthy humans, about one-third of glucose is taken up by the liver[21]. Estimates of skeletal muscle glucose uptake vary widely, in part because they are often based on indirect measurements and assumptions regarding muscle mass and blood flow. One report that measured muscle glucose more directly using nuclear magnetic resonance demonstrated muscle absorbing 64%-91% of infused glucose in a single male volunteer[22]. A follow-up study of 11 subjects reported muscle glucose uptake of 90% in normal subjects and 67% in diabetic subjects[23]. In a separate study of 10 healthy volunteers, muscle accounted for 38.3% of systemic glucose disposal, based on data from blood sampled from a forearm vein[24]. Overall, the data show greater glucose uptake in skeletal muscle than liver in humans. Genetic evidence underscores the importance of skeletal muscle to whole-body glucose tolerance in humans. Polymorphisms in the gene for the primary glucose transporter in muscle, glucose transporter isoform 4 (GLUT4), have been linked to type 2 diabetes and insulin resistance[25]. Overall, defects in skeletal muscle glucose disposal are a major component of insulin resistance in humans[26].

By contrast, the liver is much more important for glucose disposal in mice. Interfering with glucose uptake in mouse liver causes whole-body insulin resistance and glucose intolerance, but similar manipulations in muscle usually do not. The muscle-specific insulin receptor knockout mouse has normal glucose tolerance, insulin sensitivity, and glucose and insulin levels, with only mild dyslipidemia[27]. Muscle-specific deletion of IRS1 and IRS2 also does not produce a diabetic phenotype, nor does a whole-body knockout of the major muscle glucose transporter, GLUT4[28,29]. One exception to this pattern may be a muscle-specific GLUT4 knockout strain that developed a diabetic phenotype in one study[30], a result that has not been replicated by others[31,32]. In contrast to the above strains deficient in muscle insulin signaling, a liver-specific insulin receptor knockout mouse strain was insulin resistant and severely hyperinsulinemic, and developed hyperglycemia and glucose intolerance at an early age (2 mo)[33]. Liver-specific deletion of IRS1 and IRS2 also cause insulin resistance under certain conditions[34]. Mice with a deletion of the primary glucose transporter in the liver, GLUT2, are hyperglycemic and die at 2-3 wk of age[35].

Glycogen storage is a major destination for glucose in mammals. In mice, approximately 8 times more glyco-

gen is stored in the liver than skeletal muscle[36], but the reverse is true in humans, where 3-8 times more glycogen is found in skeletal muscle[37]. These physiological differences in glucose disposal and storage have implications for modeling insulin resistance, since muscle and liver have different roles and different metabolic and signaling pathways.

There are two important differences in glucose transport between liver, the primary glucose disposal organ in mice, and skeletal muscle, the primary glucose disposal organ in humans. First, skeletal muscle cells have multiple pathways for glucose transport. Contraction-stimulated glucose transport in skeletal muscle is insulin-independent, mediated through 5' adenosine monophosphate activated protein kinase-mediated signaling mechanisms[38]. In contrast, liver has no such activity-stimulated transport method. Second, the transporters involved in glucose uptake are different in the two tissues. In liver, the low-affinity GLUT2 is present at high levels on cell membranes independent of insulin or other signaling[39], and glucose transport rates vary with the extracellular concentration of glucose[40]. In contrast, in skeletal muscle cells, the high-affinity glucose transporter GLUT4 is translocated from internal vesicles to the plasma membrane in response to glucose uptake signals[41]. In human skeletal muscle cells, this transport is facilitated by clathrin isoform CHC22, which is not present in the mouse[42]. The rate-limiting step in glucose metabolism in liver is phosphorylation, while in skeletal muscle it is transport through GLUT4[43]. The divergent features of cells in these organs, combined with the divergent physiology of rodents and humans, means that glucose disposal is affected very differently in the different species.

Because mice rely principally on the liver for glucose homeostasis, while humans rely on skeletal muscle where transport mechanisms and biochemical pathways differ, mice may not be expected to be analogous to type 2 diabetes patients in regards to mechanisms of glucose metabolism or its dysfunction.

Mice and humans have a number of other metabolic differences. The small size and fast metabolism of mice enables heart rates in the range of 350-550 beats per minute, while in humans, normal heart rate is about 70 beats per minute[44]. Mice are capable of the physiological state of torpor, a state of reduced metabolic rate, while humans are not[45]. Prolonged fasting in humans impairs insulin-stimulated glucose utilization, but causes enhancement in mice[46]. In regards to eating patterns, mice consume most of their food at night[45], and an overnight fast of 14-18 h, typical for laboratory experiments, induces a state akin to starvation[47]. In addition, circulating lipids have an inverted composition in mice, with high-density lipoprotein (HDL) being typically higher than low-density lipoprotein (LDL), while HDL is lower in humans[48]. The thermoneutrality point, that is, the temperature at which an organism expends minimal energy for temperature regulation, is higher in mice[49]. This last difference could be compensated for if mice were housed above room temperature, but that is not standard practice.

Finally, experiments investigating mouse metabolism present technical challenges. Insulin sensitivity is often measured using a hyperinsulinemic-euglycemic clamp test, which involves either implanted arterial catheters or repeated blood sampling. The results of this test are dependent on a number of experimental factors which are not standardized between laboratories, including fasting time, anesthesia use, and blood sampling site[46]. Fasting glucose, insulin, and lipid levels are often measured after 14-18 h overnight fasts, but this induces a catabolic state in mice, who normally eat mostly at night. Data shows that a 6 h fast is best to assess glucose tolerance in mice[50].

KNOCKOUT MODELS OF INSULIN SIGNALING

Mouse models of diabetes are often used to explore signaling pathways[13]. The following sections highlight cases relevant to insulin signaling dysfunction where similar or identical genetic manipulations produced disparate results. These cases are consistent with other results showing differences in insulin action, secretion, and responses to hypoglycemia in different inbred mouse strains[51]. Previous reviewers have also noted the strong effect of genetic background in knockout mouse experiments[52]. Other factors influencing disparate findings include compensatory metabolic adjustments and technical challenges associated with evaluating mouse metabolism. Later, we will focus on the challenges of translating mouse knockout results to humans.

INSULIN RECEPTOR AND INSULIN RECEPTOR SUBSTRATE

Binding of insulin to the insulin receptor is the first step in the insulin signaling pathway. Mice with complete deletion of the insulin receptor are about 10% underweight and suffer from chronic hyperglycemia[53,54]. They die within several days of birth due to diabetic ketoacidosis. In humans, donohue syndrome is a rare monogenic disease resulting from mutation of the insulin receptor. Individuals with this disease suffer from severe pre-natal and post-natal growth retardation, fasting hypoglycemia, and post-prandial hyperglycemia[55]. They generally die before adulthood. The difference between the glucose homeostasis in mice and humans with this mutation may be attributable to the fact that the human pancreas develops earlier in gestation, hence better enables the compensatory hyperinsulinemia[55].

The pancreatic beta-cell specific insulin receptor knockout mouse strain (called BIRKO) has impaired insulin response to glucose challenge and develops impaired glucose tolerance and high insulin levels[56]. In the initial description of this mutant strain, glucose levels and body weight were normal, however, a follow-up report from the same laboratory described consistent hyperglycemia and sporadic obesity[57]. In the same report, a muscle

Table 1 Knockout mouse reproducibility

Model	Ref.	Genetic background	Observed discrepancy
IRS1 knockout	Tamemoto *et al*[19]	C57BL/6 × CBA	Growth defect twice as severe in Araki 1994
	Araki *et al*[18]	C57BL/6	
IRS2 knockout	Withers *et al*[60]	C57BL6 × 129Sv	Growth defect observed only in Withers *et al*[60]. Much more severe glucose dysregulation in Withers *et al*[60]
	Kubota *et al*[61]	C57BL/6 × CBA mixed	
IR and IRS1 double heterozygous knockout	Kulkarni *et al*[62]	C57BL/6 129/Sv DBA/2	Diabetes not observed in 129/Sv mice, observed in 85% of C57BL/6 mice and 64% of DBA/2 mice. Glucose intolerance only in C57BL/6 strain
AKT2 knockout	Cho *et al*[64]	C57BL/6	More severe hyperglycemia and hyperinsulinemia in Garofalo *et al*[63]. Growth defect only in Garofalo *et al*[63]
	Garofalo *et al*[63]	DBA/1lacJ	
AKT1 knockout	Chen *et al*[65]	C57BL/6 × 129R1	High neonatal mortality only in Cho *et al*[64]. Improved glucose tolerance and insulin sensitivity only in Buzzi *et al*[68]
	Cho *et al*[66]	C57BL/6	
	Buzzi *et al*[68]	129/Ola, C57BL/6 mixed	
Pik3r1 heterozygote	Mauvais-Jarvis *et al*[72]	129Sv, C57BL/6 mixed	Improved glucose tolerance and insulin sensitivity and low glucose and insulin levels on normal diet only in Mauvais-Jarvis *et al*[72]
	McCurdy *et al*[73]	C57BL/6SVJ	
Liver-specific *Pik3ca*	Sopasakis *et al*[74]	129Sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Sopasakis *et al*[74] only
	Chattopadhyay *et al*[75]	129, C57BL/6J mixed	
GLUT4 heterozygous knockout	Stenbit *et al*[76]	CD1, C57BL/6 mixed	Unexpected more severe phenotype in heterozygous knockout than homozygous
PKCλ heterozygous knockout	Farese *et al*[79]	C57BL/6, 129P2/Sv, FVB mixed	Unexpected more severe hepatic steatosis in heterozygous knockout than homozygous
PKCδ knockout	Leitges *et al*[81]	129/SV × Ola	High neonatal mortality observed only in Bezy *et al*[82]
	Bezy *et al*[82]	C57BL6/J	
PPARγ	He *et al*[86]	C57BL/6J	Resistance to diet-induced insulin resistance only in Jones *et al*[85] study
	Jones *et al*[85]	C57BL/6J, FVB mixed	
Muscle-specific PPARγ	Norris *et al*[87]	129/sv, C57BL/6, FVB mixed	Insulin resistance and glucose intolerance on normal diet in Hevener *et al*[88] only. Improvement with rosiglitazone in Norris *et al*[87] only
	Hevener *et al*[88]	C57BL6/J	

Reproducibility problems in knockout mouse studies. Some variant results can be explained by differences in genetic background. IRS: Insulin receptor substrate 1; IR: Insulin receptor; AKT2: Protein kinase B isoform 2; GLUT4: Glucose transporter isoform 4; PKCλ: Protein kinase C λ; PPARγ: Peroxisome proliferator-activated receptor γ.

and beta-cell double insulin receptor knockout (BIRKO-MIRKO) mouse strain had an unexpectedly mild condition. This strain had impaired glucose tolerance, mild hyperglycemia, high triglycerides and free fatty acids, and extra fat pad mass. These findings would seem to indicate that muscle-mediated glucose disposal is dispensable for normal glucose homeostasis in mice, but 2-deoxyglucose uptake studies showed that both muscle-specific insulin receptor knockout (MIRKO) and BIRKO had normal muscle glucose uptake, suggesting most muscle glucose uptake under these conditions is insulin-independent[57]. Studies of liver glycogen synthesis and liver glycogen content confirm that mice with insulin insensitive muscle shifted glucose utilization away from muscle and towards liver[57].

Mouse strains lacking insulin receptor in other tissues have been developed. A knockout of insulin receptor in neuronal tissue (NIRKO) demonstrated elevated body weight, white adipose tissue, serum triglycerides, and circulating leptin, with most of these changes being more pronounced in the females[58]. In addition, both sexes of NIRKO mice had reduced fertility, demonstrating the importance of insulin in reproduction. A knockout of insulin receptor in adipose tissue (FIRKO) had low fat mass, and the normal relationship between leptin levels and fat mass was disrupted[59]. These mice were protected against age-related glucose intolerance.

The IRS proteins transmit signals from the insulin and IGF1 (insulin-like growth factor 1) receptors. Two groups independently showed a significant pre-natal and post-natal growth defect in IRS1 knockout mice[18,19] (Table 1). Despite having similar genetic backgrounds, only one of the strains exhibited glucose intolerance as measured by a glucose tolerance test[18]. In addition, the two strains had significantly different growth defect severities, with a 40%-60% decrease in weight at various life stages observed in one study[18], and a 20%-30% decrease in the other[19]. These differences could have been due to the genetic manipulation approaches or the genetic backgrounds.

Two independent groups described IRS2 knockout mouse models, and the phenotypes were different despite similar genetic backgrounds. Withers *et al*[60] observed a 10% decrease in body weight throughout all life stages for the IRS2 knockout mice in a C57BL6 × 129Sv background, while Kubota *et al*[61] observed the IRS knockouts to be of normal size in a C57BL/6 × CBA mixed background. Fasting hyperglycemia was observed at age 6 wk in Withers *et al*[60], but average glucose levels did not reach hyperglycemic levels in Kubota *et al*[61]. Hyperinsulinemia and glucose tolerance showed a similar pattern: more severe, earlier phenotypes observed in Withers *et al*[60] than in Kubota *et al*[61]. Reduced β-cell mass was observed by both groups.

Kubota *et al*[61] suggested that the difference in glucose and insulin levels between the two reports was likely due to low β-cell mass in their strain, caused either by β-cell death or by the failure of insulin-resistance induced hyperplasia, and acknowledge that genetic differences other than the intended manipulation may influence the results. The authors concluded based on their data and data from a related study that both β-cell dysfunction and reduced β-cell mass can contribute to the murine diabetic state, but only studies of human patients can validate whether one or both mechanisms are more important in the pathogenesis of type 2 diabetes in humans.

Double heterozygous knockout of IR and IRS1 were generated in three different genetic backgrounds: C57BL/6, 129/Sv and DBA/2[62]. While all three strains had mild growth retardation, the results in regards to glucose homeostasis were drastically different. In C57BL/6 mice, the double heterozygous knockout caused severe hyperglycemia and hyperinsulinemia in the vast majority of cases, whereas the glucose levels of 129Sv mice were not significantly different from control littermates. In DBA mice, more than half of the mice were hyperglycemic but maintained normal glucose tolerance. Triglycerides were significantly reduced in the double heterozygous knockouts of the B6 and DBA strains, and the wild type DBA strain had significantly elevated triglycerides as compared to the other wild type strains[62].

AKT/PROTEIN KINASE B

The metabolite phosphatidylinositol 3,4,5-trisphosphate (PIP3) activates AKT/protein kinase B and atypical protein kinase C. AKT has three isoforms in mammals, of which AKT1 and AKT2 are most important for metabolism. Two independently developed AKT2 knockout mouse strains in different backgrounds developed hyperglycemia, glucose intolerance, and insulin resistance[63,64]. Garofalo *et al*[63] observed hypoinsulinemia due to pancreatic β-cell death in a subset of male mice, and hyperinsulinemia with no pancreatic changes in the remainder, while Cho *et al*[64] observed hyperinsulinemia and associated pancreatic hyperplasia. In Garofalo *et al*[63], both hyperglycemia and hyperinsulinemia were more severe than in Cho *et al*[64], with average fed insulin measurements five times higher. Also, Cho *et al*[64] observed normal growth in the AKT2 knockout, but Garofalo *et al*[63] observed a mild growth deficiency evident at all life stages. Only Garofalo *et al*[63] observed lipoatrophy and high levels of serum triglycerides. The control mice in Garofalo *et al*[63] had near-diabetic random fed glucose levels that were almost as high as the knockout mice in Cho *et al*[64] Neither of these knockout strains were obese.

The characteristics of AKT1 knockout mouse strains are also sensitive to genetic background and environmental factors. Two labs independently reported that AKT1 knockout mice with different genetic backgrounds had a growth defect causing 15%-20% reduced body weight[65,66]. One of the studies observed high neonatal mortality among the knockout mice[66], while the other observed high mortality with γ-radiation[65]. Glucose tolerance in Chen *et al*[65] appeared normal, but the glucose tolerance test was performed using a longer fasting time and lower glucose dose than is optimal[50]. One study demonstrated a non-significant improvement in glucose tolerance and insulin sensitivity in males. A similar strain was later shown to be resistant to diet-induced obesity[67]. Later data on a third, independently developed AKT1 knockout strain showed dramatic improvement in glucose tolerance and insulin sensitivity[68].

Studies of spontaneous human genetic variants in AKT1 and AKT2 have confirmed the importance of these proteins in growth and glucose homeostasis, mostly respectively, although the manifestations of the mutations differ between humans and mice[16]. For example, the human patients with a specific AKT2 mutation display asymmetric hypertrophy[69], while the above-described AKT2 knockout mouse models have normal growth[64] or a growth deficiency[63].

PHOSPHOINOSITIDE 3-KINASE

PI3K, an enzyme complex composed of a regulatory subunit and a catalytic subunit that produces the metabolite PIP3. PI3K is activated by IRS proteins in the insulin signaling cascade (Figure 2). In humans, *PI3K* gene polymorphisms are associated with cancer risk[70] but not diabetes, to our knowledge.

Complete loss of the *Pik3r1* gene, which encodes isoforms of the regulatory subunit of PI3K, results in perinatal lethality in mice, perhaps due to impaired B cell development[71]. Mice heterozygous for *Pik3r1* deletion, having attenuated expression of all isoforms of the regulatory subunit, had improved glucose tolerance and insulin sensitivity and low glucose and insulin levels[72]. Lipid metabolism was unchanged except for a modest increase in serum free fatty acids, indicating that the observed insulin sensitivity was not due to indirect effects *via* changes in lipid metabolism. A minor increase in basal muscle glucose uptake was observed, but the authors note that changes in liver were likely most responsible for the increased insulin sensitivity[72]. A later, independent study observed that the heterozygous knockout mice were essentially indistinguishable from control mice on a normal diet[73]. On a high-fat diet, these mice showed lower fasting insulin levels, improved overall insulin sensitivity, and improved glucose uptake in fat and muscle[73]. Macrophage accumulation was reduced in the adipose tissue of these heterozygous knockout mice, but results from bone marrow transplant experiments suggested the improved insulin sensitivity did not occur solely *via* PI3K's role in inflammation.

The catalytic subunits of PI3K have also been studied using knockout mouse strains. Liver-specific deletion of *Pik3ca* caused mild obesity, insulin resistance, glucose intolerance, and high glucose and insulin levels[74]. The same genetic manipulation in a second laboratory produced a strain with normal glucose and insulin levels and body weight[75]. The *Pik3ca* knockout mice in the second

study were resistant to high-fat diet induced hepatic steatosis and somewhat resistant to diet-induced glucose intolerance as well[75]. For this gene, liver-specific deletion produced diabetes-like symptoms in one laboratory, but in another laboratory, glucose homeostasis was identical in control and knockout mice[74,75].

GLUT4

As described above, GLUT4 is the major glucose transporter in muscle, the most important tissue type for glucose disposal in humans. Unexpectedly, in GLUT4 knockout mice, glucose levels are normal except for mild fed hyperglycemia and fasted hypoglycemia observed only in males[29]. Consistent with results regarding insulin signaling and growth[18], these animals display significant growth retardation, shortened life spans, cardiac hypertrophy, and reduced adipose tissue[29]. Somewhat surprisingly, mice heterozygous for the GLUT4 knockout have a more severe phenotype. A diabetes-like condition developed at varying ages, with a majority of males both hyperinsulinemic and hyperglycemic by age 5-7 mo[76].

The authors pointed out that the unexpectedly mild condition of the homozygous GLUT4 knockout and more severe condition in the GLUT4 knockout heterozygote were likely due to compensatory metabolic adjustments that occur during development. These could include the transfer of glucose disposal from tissues that primarily use GLUT4 to tissues that primarily use GLUT2, as observed in the muscle-specific GLUT4 knockout[30], or the upregulation of alternative glucose transporters[52].

PROTEIN KINASE C

Protein kinase C enzymes (PKCs) are involved in regulating a variety of cellular functions in mammals, including insulin signaling[77]. Atypical PKCs include the isoforms PKCλ/ι and ζ (PKCλ refers to the mouse isoform of PKCι)[78]. Activated PKCs can inhibit insulin signaling by a feedback mechanism that prevents signal transduction between insulin receptor and IRS[7,78].

Atypical protein kinase C family member PKCλ was knocked out specifically in mouse muscle, resulting in diabetic symptoms including glucose intolerance, insulin resistance, hyperglycemia, and high insulin levels[79]. Altered fat metabolism was also observed: high triglycerides, and mildly elevated free fatty acids and liver triglycerides. While some symptoms were observed in both the heterozygous and homozygous muscle-specific knockout of PKCλ, the heterozygotes were as insulin resistant and glucose intolerant as the homozygous knockouts, and had more abdominal obesity and hepatic steatosis[79]. This is unexpected, since the heterozygous knockout had reduced, but not ablated, expression of PKCλ.

Differential expression of PKCδ has been identified as one factor in the different vulnerability of common laboratory mouse strains to diabetes[80]. One study of a PKCδ knockout mouse strain in a 129/Sv × Ola genetic background had normal growth and development[81]. Surprisingly, the same deletion in the C57BL6/J strain caused a high mortality rate, with survivors being 14% underweight[82]. The C57BL6/J PKCδ knockout mouse had better glucose tolerance than control mice[82], but glucose tolerance was not tested in the original knockout. The authors noted that improved glucose tolerance may have been due to decreased inflammation in adipose tissue[82]. In humans, PKCδ deficiency can cause B-cell deficiency with severe autoimmunity[83].

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ

The nuclear receptor PPARγ, becomes activated upon binding of lipids and is important for lipid metabolism and storage, adipogenesis, and insulin sensitivity. This nuclear receptor is the target of insulin-sensitizing TZDs[84].

Two independently generated adipose tissue-specific PPARγ knockout strains showed important differences in glucose homeostasis under high-fat diet conditions. On normal chow, both these strains had reduced adipose tissue mass, high blood lipid levels, and hepatic steatosis, but glucose tolerance was normal[85,86]. On high-fat diet with 40% of calories from fat, He *et al*[86] observed hyperinsulinemia and insulin resistance in both the knockout and control mice, although these traits were more severe in the knockout. The knockout strain studied by Jones *et al*[85] was resistant to diet-induced hyperinsulinemia and insulin resistance despite being subjected to a more extreme high-fat diet, with 60% of calories from fat. The knockout strains in both studies were more prone to high-fat diet induced hepatic steatosis.

Two studies on independently developed muscle-specific PPARγ knockout models have provided contradictory findings regarding the mechanism of action of TZDs. The first strain was more susceptible to diet-induced obesity, glucose intolerance, and insulin resistance but was indistinguishable from controls on a normal diet[87]. Rosiglitazone reduced the hyperinsulinemia and impaired glucose homeostasis observed in this strain on high-fat diet, therefore the authors suggested that muscle PPARγ is not required for the positive effects of this TZD[87]. In contrast, the second strain developed insulin resistance and glucose intolerance on a normal diet[88]. Glucose disposal in a hyperinsulinemic-euglycemic clamp experiment was not improved with rosiglitazone treatment, suggesting that the insulin sensitizing effect of TZDs is dependent on muscle PPARγ. In this case, two mouse models have provided conflicting data not just on the role of a gene, but also on a drug mechanism of action.

In conclusion, we above described several cases where genetic modification of insulin signaling genes produced significantly, sometimes dramatically, different results in separate studies or varied genetic backgrounds (Table 1). We also described two cases where heterozygous knockouts had unexpectedly severe phenotypes: GLUT4 and

PKCλ. Although the mechanisms behind the unexpected observations are unknown, it is known that organisms respond unpredictably to the absence of gene products during development. Compensatory metabolic adjustments that may occur during development constitute a general limitation of knockout mouse models. These concerns are mitigated by the use of conditional knockouts, however, those strains require injection or gavage of an inducing drug, which can produce artifacts[89]. These examples illustrate the challenges associated with producing reliable, reproducible, and translatable results in mice.

CLINICAL TRANSLATION

In the following section, we will address factors which limit the applicability of mouse models to human therapeutic treatment development. As described above, insulin signaling gene knockout mice often have phenotypes unrelated to type 2 diabetes including growth defects[18,33,60,63], neonatal mortality[66], and others, including resistance to tumor formation[90]. These phenotypes are a result of the loss of diverse non-metabolic insulin functions, and these studies have yielded information about those biological processes in mice. At this juncture, it is worth examining whether these mouse models of insulin resistance are contributing positively to the development of new, unique, safe, and effective type 2 diabetes treatments. Here we focus on select pharmaceuticals targeting the signaling proteins discussed above.

As might be predicted based on the importance of insulin to growth, several drugs targeting insulin signaling molecules PI3K and AKT are under investigation as therapeutics for cancer[91,92]. Unsurprisingly, some PI3K inhibitors have been shown to induce insulin resistance[93].

The nuclear receptor PPARγ is an important drug target, and is genetically linked to insulin sensitivity and type 2 diabetes risk[94,95]. However, PPARγ-activating TZD drugs are associated with a number of side effects and risks, including congestive heart failure[96]. Although some studies have been inconclusive in regards to certain risks associated with the TZD rosiglitazone[97], one meta-analysis of 42 studies found that the risk of cardiovascular death increased 64%[98]. Rodent studies did not predict these deaths, and in fact have provided conflicting evidence regarding cardioprotective and cardiotoxic effects of TZDs. The TZD pioglitazone was shown to limit myocardial infarct size after coronary occlusion in mice[99]. Similar results have been seen for rosiglitazone after ischemia/reperfusion injury[100]. TZDs have been shown to have both positive and negative effects on cardiac hypertrophy in rodents[101,102].

An inhibitor of PKCβ, LY333531, or ruboxistaurin, has been investigated as a potential treatment for diabetic microvascular complications[103]. Although initially promising results were observed in a trial for diabetic neuropathy, the drug was not shown to be effective in a larger, placebo-controlled study[104]. Promising results were also seen in a small trial for diabetic kidney disease[105], but these have not been replicated at a larger scale. Eli Lilly withdrew the marketing authorization application for ruboxistaurin as a treatment for diabetic retinopathy. Rather than diabetes or its complications, PKC inhibitors are now being investigated as potential treatments for cancer[106] and conditions requiring immunosuppressive therapy[107].

CONCLUSION

The limitations of these mouse models of insulin signaling dysfunction arise from a number of sources. Described above are physiological and molecular-level differences between mice and humans, reproducibility problems in mouse experiments, and complicating factors in drug discovery efforts that interfere with translating mouse results to human patients.

Researchers in a variety of fields have commented on the limitations of mouse models of human disease[108,109]. No single mouse model can accurately represent the spectrum of symptoms and complications associated with type 2 diabetes[11]. The translation of results from mice is further complicated by a plethora of immutable species differences at every level of glucose regulation from the molecular to the population level[110-113]. In addition, mice are not prone to hypertension, high LDL cholesterol, atherosclerosis, sedentary behavior, obesity, insulin resistance, or many other features common to human type 2 diabetes patients. Although all laboratory mice are more insulin resistant and have more fat tissue than their free-living counterparts[114], the risk for mice developing these symptoms varies widely depending on the specific inbred strain[62,80]. Genetic background, housing conditions, and diet can dramatically affect results. Examples highlighted here have shown that different studies even from the same laboratory often obtain different results with identical genetic modifications.

The idea that the limitations of genetically modified mouse models of human disease, and rodent models in general, are severe enough to warrant a shift in research approaches is controversial, and will likely continue to be for the next decade. Nonetheless, science in many medical fields has been progressing away from crude, animal-based experiments and towards more high-tech and human-based research methods, and that trend will continue. For example, one area of active research is additional uncharacterized insulin signaling cofactors, which could be identified using phosphoproteomics[115], protein array techniques, or protein interaction-based techniques[116] including yeast two-hybrid and computational approaches. Similar approaches could be used to identify gene products involved in acquired insulin resistance. In addition, insulin resistance can be investigated in human cells by gene silencing[117], metabolomics[118], and microarray technology. Remaining questions about the role of inflammation and accumulated intracellular lipids can be studied using tissue biopsy samples from various patient populations[119]. Many more *in vitro*[120], in silico[121], non-invasive[122], and minimally invasive[123] approaches are available and in development.

In the last 20 years, the use of genetically modified mice to investigate diabetes has become routine. While some findings have borne out in humans, investigations of insulin resistance using knockout mouse models are inherently limited by physiological, genetic, and metabolic differences between mice and humans. Researchers and patients would benefit from a transition towards human-based research methods.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Drs. Mark Atkinson, Morris Birnbaum and John J. Pippin for critical reading of the manuscript. The authors thank Doug Hall for graphic design.

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P- Reviewers: Nakhoul FM, Sasaoka T **S- Editor**: Song XX
L- Editor: A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Transdifferentiation of pancreatic α-cells into insulin-secreting cells: From experimental models to underlying mechanisms

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Supported by The Agence National de Recherche (ANR 2010 BLAN 1240 01), the Ligue contre le Cancer du Rhône and de la Loire; the CMIRA program of Region Rhône-Alpes, No. 12004959-01; the Fondation ARC pour la Recherche sur le Cancer, France, No. SFI20101201530; National Nature Science Foundation of China, No. NSFC81170719, No. 81370960; and Shanghai New Excellent Youth Program, No. XYQ2011009
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Received: November 27, 2013 Revised: July 19, 2014
Accepted: July 27, 2014
Published online: December 15, 2014

Abstract

Pancreatic insulin-secreting β-cells are essential regulators of glucose metabolism. New strategies are currently being investigated to create insulin-producing β cells to replace deficient β cells, including the differentiation of either stem or progenitor cells, and the newly uncovered transdifferentiation of mature non-β islet cell types. However, in order to correctly drive any cell to adopt a new β-cell fate, a better understanding of the *in vivo* mechanisms involved in the plasticity and biology of islet cells is urgently required. Here, we review the recent studies reporting the phenomenon of transdifferentiation of α cells into β cells by focusing on the major candidates and contexts revealed to be involved in adult β-cell regeneration through this process. The possible underlying mechanisms of transdifferentiation and the interactions between several key factors involved in the process are also addressed. We propose that it is of importance to further study the molecular and cellular mechanisms underlying α- to β-cell transdifferentiation, in order to make β-cell regeneration from α cells a relevant and realizable strategy for developing cell-replacement therapy.

Key words: α-cell; β-cell; Transdifferentiation; Diabetes mellitus; Cell-replacement therapy

Core tip: Recent works highlighted the phenomenon of transdifferentiation of pancreatic α cells into β cells, which has drawn much attention in the field. Considering that α-cell transdifferentiation could be used as a new strategy of cell replacement therapy for the treatment of diabetes, because of the presence of α cells in the pancreas of both type 1 and 2 diabetics, we believe that it is relevant to elucidate the cellular and molecular events in α- to β-cell conversion. Our review focuses on the recent experimental α-cell transdifferentiation models, highlighting the insight provided by these works into the candidates and contexts revealed to be involved in this process.

Original sources: Lu J, Jaafer R, Bonnavion R, Bertolino P, Zhang CX. Transdifferentiation of pancreatic α-cells into insulin-secreting cells: From experimental models to underlying mechanisms. *World J Diabetes* 2014; 5(6): 847-853 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/847.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.847

INTRODUCTION

Pancreatic β cells are vital for glucose homeostasis. They are capable of producing and secreting insulin, a peptide hormone, in respond to high blood glucose levels. Insulin acts on diverse tissues to stimulate the metabolism of glucose[1,2]. Diabetes mellitus, becoming an epidemic in different parts of the world and a major public health challenge, is a carbohydrate metabolic disorder arising from failure of glucose homeostasis, with consequent hyperglycemia, resulting in severe complications affecting numerous tissues. The International Diabetes Federation estimated that 336 million individuals worldwide had diabetes in 2010. By 2030, this will have risen to 552 million[3]. The disease is characterized by either defective β-cell function as seen in Type 1 diabetes patients who have insufficient or even no β cells, or increased insulin resistance as observed in Type 2 diabetics who fail to maintain glycemic control because of, at least partially, insufficiency in β-cell mass or function. Consequently, there is an urgent need to search for efficient strategies to generate functional β-cells for cell replacement therapy.

The current strategies of generating new β cells can be outlined mainly in the following three ways[2,4]: (1) pluripotent stem cell differentiation: with the combined use of different factors, a pluripotent stem cell can be directed to differentiate into the cells with insulin-producing capability. Although such a directed differentiation seems to mimic normal pancreatic development, functional β cells can currently only be differentiated through a lengthy transplantation step; (2) inducing cell replication in existing β cells: this may be conducted either *in vitro* or *in vivo* using different agents or factors, but caution should be taken to avoid neoplastic transformation; and (3) reprogramming a differentiated cell by using genetic factors to induce a pluripotent state and factors driving a specific differentiation program. Reprogramming of acinar cells to generate β cells has proved to be successful *in vivo*[5]. More recently, a new strategy, the transdifferentiation of fully differentiated α cells into β cells, has emerged.

Transdifferentiation was originally defined as the change in a given adult cell from its initial differentiated state into another[2]. The most well known cell transdifferentiation phenomenon comes from the regenerative ability seen in urodele amphibians, which can regenerate their limbs, jaws, lens and large sections of their hearts. It is generally thought that transdifferentiating cells may go firstly through dedifferentiation, then proliferation and finally redifferentiation stages. Transdifferentiation can be distinguished from the above-mentioned directed stem cell differentiation by the fact that the initial cells are not "undifferentiated". Consequently, transdifferentiated cells are not systematically clonogenic. Although different examples of transdifferentiation were cited[2], it remains uncertain whether "natural" transdifferentiation can actually occur in mammals. More interestingly, recent studies have reported several experimental transdifferentiation models triggered either by drastically changing cellular and/or tissue contexts, or by directly altering molecular programs governing the cellular differentiation state (often referred to as cell conversion). Most notably, it is known that acino-ductal transdifferentiation can be seen in the case of severe tissue injury in the pancreas[6,7]. The treatment of rats with a copper-deficient diet resulted in the appearance of hepatocytes in the pancreas, whereas a reversed transdifferentiation was observed in the treatment of rats with polychlorinated biphenyls[8]. Experimental works have shown that either the pancreatic acinar tumor cell line AR42-J[9], or freshly isolated adult acinar cells[10] can transdifferentiate into hepatocytes *in vitro*. It was also reported that, under certain cell culture conditions, AR42-J cells were seen to display endocrine cell features[11,12]. Similarly, with the use of epidermal growth factor- and leukemia inhibitory factor-supplemented cell culture medium, it was reported that pancreatic exocrine cells were transdifferentiated into insulin-producing cells[13]. The phenomenon may also occur *in vivo*, the cells coexpressing transiently exocrine and endocrine markers being observed in rats that were subject to duct ligation[14-16], and in mice treated with alloxan[17]. Considering the particular role of Ngn3, its ectopic expression has been explored to trigger transdifferentiation of adult human duct cells into endocrine cells[18]. Finally, it is also speculated that β-cell mass increase seen in rats chronically infused with glucose may imply transdifferentiation as mechanisms of adaptation[19,20].

More interestingly, several laboratories have reported the phenomenon of transdifferentiation of pancreatic α cells into insulin-secreting cells (Table 1), which has been observed in different experimental settings[21-36]. Because of the close developmental and physiological relationship between these two cell lineages, and the presence of α cells in the pancreas of Type 1 and 2 diabetes patients, α-cell transdifferentiation draws much attention in the field of β-cell regeneration. Here, we review in detail these different models.

EXPERIMENTAL MODELS DISPLAYING β-CELL TRANSDIFFERENTIATION

Altered cross-regulatory circuit between Arx and Pax4

A number of studies have demonstrated that, during development, the influence of several transcription factors successively directs progenitor cells toward pancreatic, and ultimately islet endocrine cell fates. A complex network of transcription factors, including Arx and Pax4, progressively and differentially promotes particular endocrine fates[21,22]. In mice lacking Arx, β- and δ-cell

Table 1 List of some experimental models of α-cell transdifferentiation

Experimental model	Phenotype	Intermediate cells	α-cell proliferation	Ref.
Pax4 overexpression	Converts progenitor cells into α and subsequently β cells	Very few	-	[24]
Arx inactivation	α- to β-like conversion	+	-	[25]
Arx inactivation; Pdx1;Arx double mutant	α- to β-like conversion	+	-	[26]
Pdx1 overexpression	α- to normal β cell conversion	Numerous mantle-located Gcg + Ins + cells were detected in P1-P12	-	[28]
PDL + alloxan	A large number of new β cells arising from adult α cells within 14 d	58% of Ins+ cells coexpressed glucagon	-	[30]
Extreme β-cell loss	α- to β-cell transdifferentiation	+	-	[29]
Treatment with histone methyltransferase inhibitor	α- to β-cell conversion	Colocalization of both glucagon and insulin in human and mouse islets	-	[36]
Ablation of glucagon gene	Normoglycemia and hyperplasia of pancreatic α cells	+	+	[31]
Ablation of glucagon receptor ($Gcgr^{-/-}$)	Lower blood glucose, hyperglucagonemia, and pancreatic α-cell hyperplasia	Few scattered Gcg + Ins + cells or not mentioned	+	[27,32-34]
Impaired glucagon synthesis ($SPC2^{-/-}$)	Normoglycemia, hyperplasia of pancreatic α and δ cells	Not mentioned	+	[37]
Disturbed glucagon pathway [Liver-specific G(s)alpha deficiency]	Hypoglycaemia, hypoinsulinemia, pancreatic α-cell hyperplasia		+	[38]
Men1 inactivation	α-cell transdifferentiation, α-cell hyperplasia and development of glucagonoma and insulinoma	+	+	[39]

fates were found to be favored at the expense of α-cell genesis, while the total endocrine cell content remained normal[21]. Conversely, in the absence of Pax4, β-cell loss was observed accompanied by an increase in α-cell number[22], indicating an inhibitory, cross-regulatory circuit between Arx and Pax4[23].

Interestingly, Collombat *et al*[24] demonstrated that ectopically expressed Pax4 in endocrine precursor cells and α cells in the mouse resulted in the conversion of these cells into insulin-producing cells. As early as 1 wk postpartum, a 50% enlargement in islet size was outlined, with the islets containing increased numbers of insulin- and Pax4-positive cells compared with controls, and the number of glucagon-producing cells reduced by 77%. An age-dependent increase in islet size and the number of insulin-producing cells was observed. The latter exhibited most β-cell features, suggesting that, upon Pax4 ectopic expression, adult glucagon-expressing cells were continuously converted into cells exhibiting a β-cell phenotype. The lack of glucagon-producing cells resulted in an apparent adaptive neogenesis of α cells. The authors provided evidence suggesting that such a conversion triggered by Pax4 ectopic expression in α cells was sufficient to alleviate the diabetic condition resulting from massive β-cell destruction in the mouse.

More recently, Wilcox *et al*[25] showed that ablation of Arx in neonatal α-cells resulted in an α-to-β-like conversion through an intermediate bihormonal state, while short-term ablation of Arx in adult mice did not. However, Courtney *et al*[26] showed that selective *Arx* disruption in α cells at any age could elicit the conversion. It is important to note that such a conversion induced duct-lining precursor cells to differentiate to endocrine cells. The α cells thus generated were subsequently converted into β-like cells because of Arx inactivation. Using conditional *Arx* and *Pax4* double mutants, Courtney *et al*[26] provided evidence showing that Pax4 was dispensable for this regeneration process, suggesting that Arx could be the main trigger of α-cell conversion into β-like cells. Importantly, Arx disruption in α cells was able to reverse mouse diabetes resulting from β-cell depletion.

α to β cell reprogramming by forced PDX1 expression

Vuguin *et al*[27] performed ectopic Pdx1 expression from Ngn3-positive endocrine progenitors (*Neurog3*^{Cre}-*Pdx1*^{OE} mice). They detected a slight increase in β-cell number accompanied by a reduced α-cell number during the embryonic period[28]. At each stage, the combined number of α and β cells in *Neurog3*^{Cre}-*Pdx1*^{OE} mice was similar to that in controls, despite a significant difference in the α- to β-cell ratio, strongly suggesting a scenario of lineage diversion, where one cell population expands at the expense of the other under a constant total cell number. Two phases of lineage conversion were identified, contributing to a complete α-cell loss by the early adult stage. First, a significant decrease in glucagon-positive cell number (47% in the control reduced to 35% in mutant mice) and accompanying increase in insulin-positive cells was detected in the E16.5 *Neurog3*^{Cre}-*Pdx1*^{OE} pancreas, shortly after the peak of Neurog3 expression at approximately E15. Second, a major progressive loss of glucagon-positive cells in parallel with increased insulin-positive cell numbers was detected at P1-P12. Coexpression of insulin and α-cell-specific factors such as Arx, suggesting an early movement toward β-cell-directed transdifferentiation, was not detected at the first stage. Importantly, numerous mantle-located glucagon- and insulin-positive cells were detected in the second stage, representing intermediate

state α cells undergoing conversion, suggesting that the suppression of glucagon and the induction of insulin occurred concurrently. Intriguingly, when activating Pdx1 in the differentiated or mature glucagon-expressing α cell, the efficiency of the occurrence of α-to-β conversion was very much impaired, even absent. The work suggests that Pdx1 alone may play a strong role in regulating the cell differentiation program of islet-cells.

Near complete β-cell ablation

Thorel *et al*[29] have generated an elegant mouse model which allows nearly total β-cell ablation using the diphtheria toxin receptor system. The massive β-cell destruction thus obtained resulted in heterologous β-cell formation. Surprisingly, the majority of newly formed β cells originated from former glucagon-producing cells. By using cell lineage tracing, they demonstrated that, upon near total loss of β cells, genetically marked α cells rapidly began firstly to coexpress Nkx6.1, then coexpress insulin and the adult β-cell markers Pdx1, Nkx6.1 and Glut2, subsequently forming the majority of the regenerated β cells. Importantly, when α cells were ablated together with β cells, bihormonal cells expressing both glucagon and insulin were no longer observed. The work may also suggest that, in this particular experimental setting, a complete lack of local insulin signaling would elicit the interconversion between α- and β-cells. It would be interesting and challenging to use this model to further study the process and the mechanisms of α-cell transdifferentiation.

Pancreatic duct ligation + alloxan treatment

Chung *et al*[30] generated another pancreas and β-cell-deficient mouse model to study the origin and extent of adult β-cell regeneration. To this end, they used the β-cell specific toxin alloxan to ablate β cells, and, subsequently, carried out pancreatic duct ligation (PDL) to stimulate β-cell neogenesis. They reported that more than half (58%) of insulin-positive cells coexpressed glucagon one week after PDL and alloxan treatment. Moreover, they found that some glucagon-positive cells coexpressed β-cell-specific transcription factors, such as Pdx1 and Nkx6.1, suggesting a transitional stage during the conversion. Later, cells coexpressing insulin and glucagon were found. Interestingly, these insulin-positive cells expressed MafB, but afterward switched from MafB to MafA expression, suggesting that they were initially immature, and became mature over time. Unfortunately, cell lineage tracing was not performed in this model.

Glucagon pathway deficiency models

Mice with glucagon signaling deficiency, due to the inactivation of either the *Glucagon* gene[31] or its receptor (*GCGR*)[27,32-34], impaired glucagon synthesis[37], or a disturbed glucagon pathway[38], display common features. These include lower blood glucose levels, improved glucose tolerance with relatively normal insulin levels, and, in particular, α-cell hyperplasia and even tumorigenesis[33], accompanied by hyperglucagonemia and, in some of these models, scattered intermediate cells coexpressing insulin and glucagon. However, full transdifferentiation of α cells into β cells has never been demonstrated in the above models. Most probably, the fact that islets were often clustered near ductal tissue, and glucagon staining was seen along and budding from ductal epithelium or within exocrine tissue, suggests that the islet neogenesis could be the cause of increased α-cell mass.

Transdifferentiation from α cells to insulin-expressing cells triggered by Men1 disruption

In our previous study, we demonstrated the phenomenon of transdifferentiation in a mouse model where the *Men1* gene, a tumor suppressor in many types of endocrine cells, is specifically disrupted in pancreatic α cells[39]. Our analyses of pancreata from aging mutant mice showed that, in spite of the α-cell specificity of the *GluCre* transgene, both glucagonomas and insulinomas, as well as mixed islet tumors, were observed in mutant mice older than 6 mo of age. More interestingly, starting from as early as 2 mo of age well before tumor onset, cells sharing characteristics of both α and β cells, and coexpressing insulin and glucagon could be identified. Importantly, using a cell lineage tracing approach, we showed that these intermediate cells and insulinoma cells were both derived from *Men1*-deficient α cells. Furthermore, our data suggest that Pdx1, MafA and Ngn3 expression did not seem to be involved in the initiation of this transdifferentiation[39]. Intriguingly, although many *Men1*-deficient α cells transdifferentiated into insulin-secreting cells, some maintained their α-cell identity. This may indicate that *Men1*-disruption *per se* does not systematically lead to α-cell transdifferentiation, but rather affords the pathophysiological conditions to allow the transdifferentiation to occur. Other factors, independent of *Men1* disruption, may, therefore, play a crucial role in the initiation of the transdifferentiation. Using this model, where transdifferentiating cells are numerous before the development of tumors, to search for these factors would be of help in further deciphering the cellular and molecular basis of α-cell transdifferentiation. The identification of such factors would be crucial to determine the conditions favorable for α-cell transdifferentiation, while avoiding the known tumorigenic effect of *Men1* inactivation in islet cells.

CLUES TO OTHER FACTORS AND UNDERLYING MECHANISMS IMPORTANT FOR TRANSDIFFERENTIATION

The data from the above mouse models displaying experimental transdifferentiation of α cells into β cells suggest that α cells could possess intrinsic abilities to allow their conversion under certain circumstances, giving rise to an adaptive response to β-cell loss or deficiency. While these

models highlighted the genetic factors directly involved in such a process, they also provided clues as to other factors that may or may not participate in α-cell transdifferentiation.

Cell dedifferentiation

It is generally considered that natural transdifferentiation occurs in two steps: the dedifferentiation of the cell, followed by the differentiation of the dedifferentiated cell into the new lineage[40]. Although it is still unclear whether experimental transdifferentiation follows a similar course, the fact that no completely dedifferentiated α cells have been reported in the above experimental models seems to indicate that this may not be the case. Instead, it may be possible to directly convert one cell type into another. In this case, there could be a simultaneous switch from the inactivation of an old cell differentiation program into the activation of a new program. The existence of "intermediate cells" expressing both glucagon and insulin documented in several of these models even suggests that the initial activation of the new program may precede the complete inactivation of the old one. However, detailed cellular and molecular analyses are still required to allow a full understanding of the transdifferentiation procedure.

Epigenetic factors

Epigenetic mechanisms are known to play an important role in establishing and maintaining cell differentiation programs. Interestingly, a recent study demonstrated that α cells harbor bivalent chromatin signatures, containing both active and repressive histone markers, at genes that are active in β cells, such as Pdx1 and MafA[36]. The finding of α-cell plasticity may be supported by the fact that β-cell specific genes are likely ready to be activated. Moreover, they found that the repressed Pdx1 and insulin expression in α cells could be reactivated by treating islets with an inhibitor of histone methyltransferase. The work provides interesting clues into eventual cell reprogramming through epigenetic modifications[36].

Along with the above data, two other studies have demonstrated that changing histone methylation marks by deleting the *Dnmt1/3a* gene resulted in the transdifferentiation of β cells into α cells[41,42]. Indeed, detailed analyses showed that these two genes, together with other epigenetic factors, such as PRMT6, MeCP2 and HDAC1, play a crucial role in inhibiting the expression of transcriptional factors that may give rise to the activation of a cell differentiation program of other cell lineages, such as ARX in β cells. The loss of DNA methylation, therefore, results in the de-repression of these transcription factors, and the activation of the transcriptional program of other cell lineages. Thus, it would be interesting to investigate whether similar mechanisms could control α-cell identity.

Islet hormones and α-cell proliferation

Glucagon, insulin and GLP1: Glucagon was found to inhibit the formation of β cells converted from α cells upon Pax4 overexpression[24]. However, the phenomenon may be more directly related to the expansion of Ngn3 progenitors rather than the reprogramming itself, since virtually all α cells were converted to insulin-expressing cells by ectopic Pax4 expression, and mutant mice displayed hypoglucagonemia. Furthermore, in the *Men1* disruption-mediated transdifferentiation model, the very high levels of glucagon did not prevent α-cell transdifferentiation[39]. As for the potential inhibitory role of insulin deduced from the work by Thorel *et al*[29], the quasi absence of insulin does not seem to be a prerequisite for the occurrence of α-cell transdifferentiation, since the majority of experimental transdifferentiation models mentioned above display substantial levels of insulin. The existence of intra-islet GLP1 in many of the experimental transdifferentiation models makes it a plausible candidate involved in α-cell transdifferentiation. However, in aged GCGR knockout mice with extremely high levels of GLP1, only α-cell expansion, likely due to neogenesis, but not transdifferentiation was observed[34]. Altogether, the above data from different experimental transdifferentiation models indicate that islet hormones themselves, including glucagon, insulin and GLP1, may not be sufficient to be critically involved in the process.

α-cell proliferation: α-cell proliferation and hyperplasia, even neoplastic changes in some circumstances, were frequently found in various glucagon-deficient models. This raises the possibility that it may be required for, or even trigger, transdifferentiation. However, in the case of GCGR knockout mice, massive α-cell proliferation and neoplastic alteration did not lead to α-cell transdifferentiation. Importantly, a patient with a homozygote germline mutation of the GCGR gene displayed microglucagonoma and non-functional islet-tumor development, but no sign of α-cell transdifferentiation[43]. The data suggest that α-cell proliferation may be favorable for, but not systematically result in, the occurrence of transdifferentiation. At the same time, this highlights the potential deleterious effects of α-cell proliferation due to drastic glucagon pathway deficiency and/or massive α-cell loss.

Timing

In a recent work reported by Wilcox *et al*[25], the authors observed that embryonic α cells and adult α cells may react differently towards *Arx* disruption. Whereas the former were driven to convert to β cells, the latter seemed completely nonresponsive to the lack of ARX. However, similar work by Courtney *et al*[26] did not confirm this observation. The reason for the discrepancy remains unclear. Interestingly, another study, using ectopic Pdx1 expression in either pancreatic progenitors or in embryonic and mature α cells to reprogram the cells into β cells, also demonstrated that the efficiency of the reprogramming decreased when forced Pdx1 expression occurred later in embryonic development or in adult mice[28]. Col-

lectively, these studies highlighted the importance of timing in α-cell plasticity that should be taken into account for possible future clinical applications based on α-cell transdifferentiation.

CONCLUSION

Taken together, the above-mentioned recent studies highlighted the importance of both transcriptional factors and/or cofactors in maintaining cell differentiation status and in the physiological mechanisms involved in α-cell transdifferentiation. It would be vital and challenging for future studies to pinpoint the decisive factors from these two axes, and to provide insight into detailed mechanisms responsible for α-cell transdifferentiation. At the same time, past experience seems to indicate that some of the above-mentioned experimental conditions, such as PLD and glucagon pathway deficiency, may be more favorable for eliciting neogenesis, rather than α-cell transdifferentiation.

Because of their close ontogenic relation with β cells and unusual plasticity in responding to internal and external alterations, pancreatic α cells elicit much curiosity and clinical promise. In particular, the capacity for their transdifferentiation into insulin-secreting cells documented by several distinct models renders them a potentially relevant cellular basis for new strategies of β-cell regeneration. Deciphering detailed cellular and molecular mechanisms of the α-cell transdifferentiation process will be challenging for the field and crucial for future clinical applications.

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P- Reviewer: Hamad A **S- Editor**: Wen LL **L- Editor**: Cant MR
E- Editor: Lu YJ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (1): Insulin

B7-H4 as a protective shield for pancreatic islet beta cells

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Received: May 29, 2014 Revised: August 16, 2014
Accepted: September 6, 2014
Published online: December 15, 2014

Abstract

Auto- and alloreactive T cells are major culprits that damage β-cells in type 1 diabetes (T1D) and islet transplantation. Current immunosuppressive drugs can alleviate immune-mediated attacks on islets. T cell co-stimulation blockade has shown great promise in autoimmunity and transplantation as it solely targets activated T cells, and therefore avoids toxicity of current immunosuppressive drugs. An attractive approach is offered by the newly-identified negative T cell co-signaling molecule B7-H4 which is expressed in normal human islets, and its expression co-localizes with insulin. A concomitant decrease in B7-H4/insulin co-localization is observed in human type 1 diabetic islets. B7-H4 may play protective roles in the pancreatic islets, preserving their function and survival. In this review we outline the protective effect of B7-H4 in the contexts of T1D, islet cell transplantation, and potentially type 2 diabetes. Current evidence offers encouraging data regarding the role of B7-H4 in reversal of autoimmune diabetes and donor-specific islet allograft tolerance. Additionally, unique expression of B7-H4 may serve as a potential biomarker for the development of T1D. Future studies should continue to focus on the islet-specific effects of B7-H4 with emphasis on mechanistic pathways in order to promote B7-H4 as a potential therapy and cure for T1D.

Key words: Diabetes mellitus; Autoimmunity; Transplantation; Co-stimulation blockade; Biomarker

Core tip: Onset of type 1 diabetes is driven by defects in immune regulation, resulting in β-cell autoimmunity. However, there may be mechanisms inherent to the β-cell that may prevent or slow development of autoimmunity and progression of disease. One such factor is B7-H4, which acts at the islet-immune interface to defend β-cells from autoimmune diabetes and to protect transplanted islet allografts.

Original sources: Sun AC, Ou D, Luciani DS, Warnock GL. B7-H4 as a protective shield for pancreatic islet beta cells. *World J Diabetes* 2014; 5(6): 739-746 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/739.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.739

INTRODUCTION

Pathophysiology of diabetes, current therapies and their limitations

Diabetes mellitus affects 382 million people world-wide today, and this number is expected to increase by 55% by 2035[1]. Diabetes is a chronic metabolic disease which stems from insufficient production of insulin by pancreatic β-cells and/or inability of the body to respond to insulin. There are two major forms of diabetes-type 1 diabetes (T1D), and type 2 diabetes (T2D). While differing in their pathogenesis, both types of diabetes result from failure and/or loss of insulin-producing β-cells that eventually translate to a state of chronic hypergly-

cemia[2-4]. Persistently high blood glucose concentrations are associated with this disease, which result in both acute metabolic conditions such as diabetes ketoacidosis and long-term vascular complications such as diabetic retinopathy, nephropathy, and neuropathy[2,4,5]. These devastating complications lead to enormous socioeconomic burdens, mandating a pressing need to find a cure.

There are both differences and similarities in mechanisms by which β-cell injuries occur in T1D and T2D. T1D has been identified as an autoimmune disease in which insulin-producing β-cells are destroyed by targeted immune attack in genetically susceptible individuals. It is believed that environmental events initially trigger the recruitment of $CD4^+$ and $CD8^+$ T cells to the islets of Langerhans and mount continuous attacks against autoantigens on β-cells, resulting in β-cell death[4,5]. T2D, closely linked to aging and obesity as well as a certain level of genetic susceptibility, is characterized by insulin insensitivity due to insulin resistance in peripheral tissues, which leads to β-cell stress[4,6,7]. T1D and T2D overlap in β-cell stress and death pathways despite differences in initiating triggers[3]. One such common pathway is endoplasmic reticulum (ER) stress, which can activate downstream signaling cascades collectively known as the unfolded protein response (UPR)[3]. Various conditions such as nutrient deprivation, inflammation, alterations in oxidation-reduction balance and elevated levels of glucose and lipids can all lead to accumulation of unfolded proteins in the ER lumen. In response to this ER stress, the UPR serves as a compensatory mechanism to restore ER homeostasis by increasing the protein folding capacity of the ER and muting protein translation[8-10]. However, chronic ER stress can shift the UPR towards a pro-apoptotic state[8,9]. In T2D, increased demand on insulin production due to progressive insulin resistance, combined with exposure to increased levels of glucose and fatty acids, induces prolonged β-cell ER stress, thus triggering cell death *via* apoptotic pathways[6,7,11]. Growing evidence also implicates ER stress as one of the factors that contribute to T1D[8,12,13]. Pro-inflammatory cytokines secreted by infiltrating immune cells in the islets of T1D patients could induce apoptosis *via* signal transducers such as STAT-1 and nuclear factor-kappa B[3,14,15], and cytokines could also negatively impact ER homeostasis and cause UPR dysregulation, which contributes to β-cell demise[3,16,17]. Knowledge of overlapping β-cell injury mechanisms between T1D and T2D can provide valuable insight into pathogenesis of diabetes, guiding rational development of therapeutics that target instigators of both T1D and T2D.

Treatments for diabetes have been designed to address glycemic control and alleviate diabetic complications. Depending on the severity of insulin resistance, management of T2D can be achieved through lifestyle and diet modifications. Commonly used pharmacological agents for T2D include insulin sensitizers, insulin secretagogues, incretin-based therapies, and insulin analogues[11]. Most T1D patients still rely on exogenous insulin injection to maintain euglycemia. However, stringent monitoring of blood glucose level is needed and the use of exogenous insulin carries the risk of hypoglycemic episodes that can be life-threatening.

In search of the elusive "cure" of diabetes, it would be desirable to halt the autoimmune attacks on β-cells, or to prevent it altogether. Current on-going clinical trials for T1D are focusing on using immunomodulation strategies to delay disease onset and preserve β-cell function in full blown diabetes. Examples of these drugs include anti-CD3 (teplizumab) and anti-CD28 (rituximab), antibodies to inhibit autoreactive T cells and B cells. CTLA4-Ig (abatacept), an inhibitory molecule for T cells, also showed promise in previous clinical trials to prolong insulin production in newly-diagnosed T1D patients[18].

Transplantation of insulin-producing tissue also provides a therapeutic option for diabetes. Whole pancreas transplantation yields better glycemic control compared with insulin injections, but subjects patients to major surgery with associated risks, and is therefore only offered to patients with severe diabetic complications. Islet cell transplantation is a relatively safe and fast alternative, in which islets isolated from cadaveric donors are infused into the liver *via* the hepatic portal vein[19,20]. With the development of the Edmonton Protocol, islet cell transplantation has become a reproducible, standardized procedure in multiple medical centers around the world which improves glycemic control[19,21]. Patients who received islet cell transplantation also showed markedly reduced diabetic retinopathy and nephropathy compared with patients who were treated with conventional medical therapy[20,21]. Even though insulin independence declined during prolonged follow up, partial graft function was maintained in 80% of the patients, as measured by C-peptide secretion[21]. Despite ongoing improvements in islet transplantation, eventual graft dysfunction, failure, and rejection remain a challenge[19,20].

The limited success of β-cell protection in various studies has attracted interest to novel β-cell immunoprotective strategies. In the following we review recent findings that suggest the negative co-stimulatory molecule B7-H4 has unique functions in the pancreatic islets that carries the potential to act as not only as a natural but also a therapeutic "shield" for β-cells during the development of diabetes and following pancreatic islet transplantation, as well its prospective role as a novel biomarker for T1D.

B7-H4: A NOVEL IMMUNE-REGULATORY MOLECULE

B7-H4, also known as B7x, was identified in 2003, and belongs to the B7 family of immunoglobulins[22-24]. Genomic B7-H4 is encoded on the *VTCN1* gene, which is located on chromosome 1 and 3 in human and mouse, respectively[24]. Given that mouse and human share 87% amino acid identity, B7-H4 is a highly evolutionarily conserved molecule. Mature B7-H4 is a 50-80 kDa transmembrane protein consisting of one IgV and one IgC

region, which are encoded on exons Ⅲ, Ⅳ, and part of Ⅴ[22-24]. Like other members of the B7 family, it is up-regulated on the cell membrane of activated antigen presenting cells, and acts to modulate the immune response[22-24]. Upon binding to a putative yet unidentified counter-receptor on T cells, B7-H4 acts as a negative co-signaling molecule to inhibit T cell proliferation and cytokine production. One proposed mechanism of action is that B7-H4 arrests cell cycle progression of T cells at the G_0/G_1 phase[23]. Since T cell activation is dependent on the presence of co-stimulatory signals, the suppressive nature of B7-H4 highlights its therapeutic potential in autoimmune diseases.

Interestingly, B7-H4 exhibits a unique mRNA profile. Unlike other B7 molecules, B7-H4 mRNA is expressed in multiple peripheral tissues such as the spleen, lung, liver, and pancreas[23]. Protein expression of B7-H4 in peripheral tissues is minimal, and its role is subject of much debate[23,25,26]. It is possible that B7-H4 undergoes tight post-transcriptional or post-translational regulation that limits its protein expression in those tissues. It remains unclear what roles B7-H4 play in the periphery, and whether it has functions that are independent of its effect on T cells. We and others have shown that the pancreas expresses moderate level of B7-H4, especially in the endocrine cells[25,27]. This raises the question of what the specific functions of B7-H4 are in pancreatic islets, and suggests the intriguing possibility that activity of B7-H4 is not limited to immune-modulation. For the purpose of this review, we will focus on the existing evidence which indicates that B7-H4 plays an essential role in islet autoimmunity and islet allotransplantation, and report data from cancer studies which alludes to other non-immune functions of B7-H4. All of the roles, known and potential, are shown in Table 1, which are classified as autoimmunity modulator, allograft protection, UPR modulation, and biomarker of β-cell immunity. This manuscript extends beyond previous reviews of B7-H4 by highlighting the importance of endogenous B7-H4 expression in β-cells, suggesting that the B7-H4 pathway for treating T1D may be more advantageous than other co-stimulatory molecules.

B7-H4 AS A PROTECTIVE SHIELD FOR β-CELLS IN T1D

Regulation of autoreactive T cells in autoimmune diseases can be achieved through various methods, such as regulatory T cell (Treg) therapy, interleukin (IL)-2 pathway manipulation, tolerance induction with antigen administration, and co-stimulation blockade[28]. As a negative co-signaling molecule, B7-H4 has the potential to down-regulate autoreactivity in autoimmune diseases such as T1D. While B7-H4 deficiency itself does not cause autoimmune diseases, various studies showed that B7-H4 plays an important role in inhibition of auto-reactive T cells in diseases such as experimental autoimmune encephalomyelitis, and rheumatoid arthritis[22,27]. Genome-wide association studies have also uncovered certain Single Nucleotide Polymorphisms within the B7-H4-encoding *VTCN1* gene as disease-causing in the context of diabetes, further implicating B7-H4 as a potential regulator of T1D[29].

Immunosuppressive functions of B7-H4 was confirmed in experimental T1D models using B7-H4-immunoglobulin (B7-H4 Ig), a recombinant protein derived from fusion of the immunoglobulin constant region to the extracellular domain of B7-H4[30,31]. Both intraperitoneal injections of B7-H4 Ig and cell-associated B7-H4 inhibited proliferation and cytotoxicity of $CD4^+$ and $CD8^+$ T cells *in vitro*[22-24,32]. Juvenile NOD mice treated with B7-H4 Ig exhibited significantly later onset as well as reduced incidence of diabetes[31]. This coincided with a reduction in proliferation and activation of both $CD4^+$ and $CD8^+$ subsets of T cells in the islet infiltrates[31]. In support of this, our preliminary findings suggested that β-cell specific over-expression of B7-H4 in transgenic NOD mice significantly decreased T1D incidence compared with wild type NOD mice (unpublished data). In conjunction with its preventive role in the onset of autoimmune diabetes, B7-H4 reversed incidence of established T1D. Return of glycemic control was observed in newly-onset diabetic NOD mice following B7-H4 Ig injections[33]. Conversely, adoptive transfer of diabetogenic T cells into B7-H4 deficient mice resulted in more exacerbated disease than wild-type controls[27]. It was hypothesized that B7-H4 did not have an effect on recruitment of immune infiltrates during the pre-diabetic stage, but rather, it prevented the progression of insulitis to overt diabetes by arresting severe insulitis at 12 wk of age in NOD mice[27,31]. This modulation of immune status at later stage of disease may be associated with down-regulation of the Th1 cells, which are widely accepted as key mediators of autoimmune diseases[31].

Mechanistic studies examining the role of B7-H4 showed that it was able to limit autoreactive CTLs, and suppressed secretion of inflammatory cytokines in the periphery[33]. For instance, levels of Th17-associated cytokines, IL-6, and IL-23, were reduced in B7-H4 treated animals[33]. This reduction was concomitant with a decrease in Th17 cells, a subpopulation of $CD4^+$ T cells that produce IL-17, IL-17F, IL-21, and IL-22, and have been implicated in various autoimmune conditions[34,35]. IL-17 is an inflammatory cytokine that may stimulate the production of other inflammatory cytokines, and is present at high levels in autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis[36-38]. Importantly, elevated Th17 cells were found in NOD mice as well as T1D patients, and were suggested to be a contributing factor to the pathogenesis of autoimmune diabetes[39-41]. One mechanism by which Th17 cells were proposed to act in T1D patients was to cause a disturbance in the ratio of T effective cell (Teff)/Treg cells, which shifted the adaptive immune response to allow development of T1D[42]. Additionally, Th17 cells were able to convert to a Th1 phenotype and stimulated cytotoxic T lymphocytes (CTL) to further contribute to autoimmunity[39]. Consistent with roles of B7-H4 in islet

Table 1 Evidence for immune regulatory and β-cell autonomous roles of B7-H4 in experimental/human diabetes

Role	Model	Summary of findings	Application	Ref.
Autoimmune modulator	NOD mouse	B7-H4 Ig inhibits development of, and reverses newly-onset autoimmune diabetes	Prevents/ reverses T1D	[31,33]
Allograft protection	NIT cell line	B7-H4 transfected NIT cells promote β-cell allograft survival	Suppresses islet graft rejection	[44]
	Mouse	Adenoviral-transduced B7-H4 donor islets enhanced islet allograft survival, and promotes donor-specific tolerance		[43,46]
	Mouse	B7-H4 transgenic islets improve islet allograft survival	Preserves β-cell mass in T1D/T2D	[51]
Non-immune dependent UPR and cell survival regulator	Pancreatic carcinoma-derived cell lines	B7-H4 knock-down increases cell apoptosis		[56]
	Renal carcinoma tissues and cancer cell lines	Human intracellular B7-H4 is identified as a cytoplasmic-nuclear shuttling protein that contains a NLS		[57]
	Mouse	B7-H4 modulates UPR in isolated pancreatic β-cells		Unpublished
Biomarkers of β-cell immunity	Mouse	B7-H4 RSS0.2 mRNA splice form is correlated with different stages of T1D	Detects β-cell autoimmunity	Unpublished
	Human	Reduced B7-H4 expression and B7-H4/insulin colocalization is detected in pancreata of T1D patients		[25]
	Human	Elevated sB7-H4 is present in RA and newly-onset T1D patients		[61,62]

T1D: Type 1 diabetes; NOD: Non-obese diabetic; UPR: Unfolded protein response; NLS: Nuclear localization signal.

autoimmunity, pancreata of B7-H4 deficient mice expressed significantly enhanced production of IL-17 and interferon (IFN)-γ, while islet-specific over-expression of B7-H4 led to a dramatic reduction in IL-17 and IFN-γ[27]. *In vitro* studies showed that cultured splenocytes displayed less affinity toward a Th17 phenotype when incubated with B7-H4 Ig, and sequestering of B7-H4 restored Th17 polarization[33]. This effect was dependent on increased IFN-γ production by the splenocytes, suggesting that inhibitory effect of B7-H4 on Th17 cell differentiation was due to stimulation of IFN-γ release[27,33]. However, it seemed that inhibition of Th17 cells by B7-H4 did not shift the Teff/Treg ratio towards Teff cells, neither did it act to expand the Th2 cell population, which is classically known as the anti-inflammatory T cell phenotype[27]. It is possible that the reduction in Th17 cells may potentially reduce the pathogenic Th1 phenotype that contributes to autoimmunity.

In summary, B7-H4 has been demonstrated to have functionality in both arresting and reversing newly-onset T1D in rodent models, and thus shows great promise as a preventative measure and a potential treatment for the disease. Current evidence suggests that B7-H4 prevents progression of severe insulitis to overt diabetes, in part, by suppressing mediators of autoimmunity such as Th1 and Th17 cells. Further research will help clarify the upstream signaling events leading to the observed beneficial effects and may significantly advance our ability to harness the potential of B7-H4 as a therapeutic for T1D.

B7-H4 INDUCES DONOR SPECIFIC TOLERANCE IN ISLET TRANSPLANTATION

B7-H4 also promotes the viability of islet grafts, and thus has significant potential for improving clinical islet transplantation as a treatment for diabetes[43-46]. Transplanted islets face many overlapping forces that conspire to limit graft function and survival, ranging from mechanical stress during isolation procedures to adverse effects of immunosuppressive drugs post-transplantation. During islet isolation and transplantation, conditions of hypoxia and nutrient deprivation collectively induce oxidative stress, ER stress and apoptosis, resulting in a decline in functional β-cell mass[47]. In the case of T1D patients, islet grafts not only encounter autoimmune surveillance, but also experience rejection mediated by alloreactive T cells. This process occurs due to priming of $CD4^+$ T cells by alloantigens presented by MHC molecules on antigen presenting cells. Activated $CD4^+$ T cells then promote the differentiation and proliferation of $CD8^+$ T cells, which attack the donor tissue. Current immunosuppressive regimens for islet transplant recipients consist mostly of tacrolimus (FK506), sirolimus (rapamycin), and mycophenolate mofetil (MMF)[20,21,48]. Generalized side effects of these drugs include increased risks for infection and malignancy, hypertension, lung toxicity, and cardiac damage. Tacrolimus has been linked to nephrotoxicity, which can be especially damaging to recipients who are at risk for diabetic nephropathy[19]. Importantly, studies have demonstrated that these drugs induced islet cell apoptosis and impaired islet function based on their mechanisms of action[19,49]. For instance, tacrolimus and sirolimus inhibit calcineurin and mammalian target of rapamycin, both of which are involved in insulin signaling and secretion[49,50]. It is therefore critical to identify novel therapeutics that offers immune-protection with minimal level of toxicity and side effects. B7-H4 is a molecule which can suppress autoimmunity as well as modulating alloreactivity, which makes it a perfect candidate for islet cell transplantation especially in T1D patients[45].

Initial investigation into the role of B7-H4 on allograft rejection demonstrated that B7-H4 protected NIT cells, a functional NOD-derived β-cell line, from injury[44]. Survival of NIT cells allotransplanted into diabetic mice was prolonged by B7-H4 transfection[44]. This was associated with reduced proliferation of recipient splenocytes,

decreased production of IFN-γ, and increased Tregs in the spleen[44]. The protective effect of B7-H4 in allotransplantation was further observed in B7-H4 adenoviral-transduced islets and B7-H4 transgenic islets. Local overexpression of recombinant B7-H4 adenovirus (Ad)-B7-H4 in intact mouse islets preserved original β-cell function and endogenous glucose responsiveness at both basal and high glucose conditions[43]. Furthermore, mice who received islets transduced with (Ad)-B7-H4 demonstrated longer allograft survival with significantly reduced infiltrates compared with control recipients[43]. Elevated Tregs and reduced cytotoxic T cells were observed in transduced islet grafts, further suggesting that B7-H4 may alter the immune environment at the graft site to induce tolerance[43]. Similarly, B7-H4 transgenic islets promoted islet allograft survival, concurrent with migration of Tregs to the graft site[51]. Tregs are known to secrete IL-10, an anti-inflammatory cytokine, and can also induce IL-10 secretion in APCs[52]. IL-10 suppresses Th1 phenotype, thus inhibiting Th1 effector cells such as $CD8^+$ T cells. In addition, Tregs also stimulated B7-H4 expression on monocytes and other APCS[52], which may act as negative co-signals to restrain T cell reactivity against donor antigens. These studies demonstrated that allotransplantation outcomes can be largely influenced by T cell co-signaling molecules, where Tregs played an important role in B7-H4 induced tolerance.

Interestingly, B7-H4 is able to achieve donor-specific tolerance rather than general unresponsiveness towards foreign antigens. When the primary B7-H4-transduced islet graft was removed and replaced with a secondary graft from the same donor mouse strain, graft survival was higher compared with a secondary graft from a third-party donor strain[46]. Isolated splenic leukocytes from recipient mice showed decreased IL-2 levels due to reduced number of IL-2 secreting cells[46]. However, no differences were observed in Tregs between mice that received same donor strain islets compared with those transplanted with third party strain islets[46]. It is possible that while Tregs are central to establishment of allograft tolerance, they may not be the main contributors to the maintenance of the secondary graft. Conceivably, B7-H4 can act on other pathways to affect IL-2 secretion and induction of donor-specific tolerance, however, this avenue of research is yet to be explored.

B7-H4 AS A DIRECT MODULATOR OF THE UNFOLDED PROTEIN RESPONSE AND CELL DEATH

The ubiquitous expression of B7-H4 in peripheral tissues has led to speculations regarding its role independent of the immune system. In support of this, studies on cancer cells reported elevated expression of B7-H4 in the cytoplasm and cell membranes from breast, uterus, and pancreas cancer cells[53-55], and its expression was correlated with tumor progression. It has been speculated that up-regulation of B7-H4 may help cancer cells evade immunosurveillance as well as being a direct tumorigenic factor independent of the immune system[56,57]. Consistent with these hypotheses, Zhang *et al*[57] demonstrated that human B7-H4 contains a nuclear localization sequence that allows B7-H4 to shuttle between the cytoplasm and the nucleus, and may regulate transcription of genes involved in cell apoptosis. Qian *et al*[56] also showed *in vitro* B7-H4 gene silencing in pancreatic cancer cells led to reduced proliferation rate and an increase in cell apoptosis that correlated with increased expression of the pro-apoptotic Bax protein and caspase activation. B7-H4 may thus play a central role in survival and apoptosis, but the exact mechanisms by which it facilitates disease progression remain an area of active investigation.

Specifically in the β-cells, endogenous B7-H4 may regulate stress *via* other cell-autonomous signaling pathways. Data from our lab suggested that *in vivo* administration of B7-H4 Ig affected the age-dependent expression of key UPR genes in the islets of NOD mice (unpublished). Notably, additional *in vitro* experiments on islets from transgenic islets with β-cell specific B7-H4 expression suggested that B7-H4 can modulate β-cell UPR signaling and may thus affect the ability of pancreatic islets to adapt to ER stress (unpublished data). In conjunction with the evidence from tumor cells, these findings support the intriguing possibility that B7-H4 also has non-immune-mediated roles in maintaining β-cell function and survival, and highlight promising new avenues for future research.

SPECIFIC EXPRESSION OF B7-H4 AS A POTENTIAL NOVEL BIOMARKER FOR T1D

While the end result of T1D is significant loss of islet β-cells that warrants the need for life-long insulin replacement, progression to end-stage diabetes occurs in several stages[58,59]. The initial step is development of islet autoimmunity, which manifests as presentation of autoantibodies to putative antigens such as GAD, ZnT8, IA-2, and insulin. Measurements of these autoantibodies have proven useful for predicting diabetes. However, after the initiation of islet autoimmunity, they are no longer able to offer consistent information regarding disease progression. From the time of autoimmunity onset to clinical diabetes there is a relatively long pre-diabetic stage. This is a critical time for therapeutic intervention, as there is theoretically still adequate functional β-cell mass at this stage of dysglycemia to preserve sufficient endogenous insulin secretion that obviates full blown T1D[60]. It is therefore vital to develop reliable markers for monitoring β-cell loss and characterizing each stage of T1D in order to determine the efficacy of therapeutic interventions associated with each stage.

In the prediction of autoimmunity, B7-H4 has been proposed to serve as a candidate biomarker for rheumatoid arthritis (RA)[55,61]. Serum samples indicated that levels of soluble B7-H4 protein (sB7-H4) in patients diagnosed with RA were significantly higher than those in

healthy donors[61]. In addition, elevated levels of sB7-H4 were associated with increased disease severity[61]. Our results showed a trend of higher sB7-H4 in diabetic children, though not statistically significant. This data agreed with a more recent study, which confirmed that sB7-H4 were elevated in newly-onset T1D patients[62]. Previous characterization of the B7-H4 gene using human multiple cDNA panels demonstrated that there are two major versions of B7-H4 transcripts from the pancreas tissue: A full-length (2.0 kb) transcript which is shared with other organs, and a shorter (1.2 kb) transcript version which is specific for pancreas[23,24]. We have also detected the presence of an additional 0.2 kb B7-H4 mRNA splicing species (RSS0.2) in the serum of T1D patients (unpublished data). Moreover, preliminary studies showed that high levels of circulating B7-H4 RSS0.2 were correlated with newly-onset T1D (< 1 year), while intermediate levels of this mRNA splice form were observed in patients with longer-term disease (1 year), and the lowest levels were found in patients with late stage T1D (2-5 years). This suggests that sB7-H4 and unique B7-H4 splice forms may serve as a novel biomarker for determining various stages of T1D.

In the human pancreas B7-H4 is more abundantly expressed in the islets than the exocrine tissue at both mRNA and protein level[25,27]. Recently, Cheung *et al*[25] showed that altered B7-H4 expression occurred in T1D and insulinoma. Multi-fluorescence immunohistochemical analyses revealed moderate expression of B7-H4 in non-diabetic pancreatic islets, significantly reduced protein expression in T1D islets, and high expression in insulinoma tumor cells[25]. Furthermore, correlation analyses demonstrated B7-H4 co-localization with insulin in both human and mouse islet[25,27]. Interestingly, the B7-H4/insulin co-localization was dramatically reduced in both T1D islets and insulinomas compared with non-diabetic islets[25]. It is possible that the reduced association between B7-H4 and insulin may reflect diseased islet states, agreeing with the observation that B7-H4 protein and mRNA expressions in islet β-cells and in sera may be useful as indicators of islet dysfunction and β-cell death/loss in the progression of T1D.

CONCLUSION

B7-H4 is the newly-identified member of the B7 immunoglobulin family commonly associated with co-stimulatory or inhibitory signals for T cells. Even though the putative receptor for B7-H4 on activated T cell is yet to be identified, its marked ability to suppress and reverse autoimmune diabetes has been demonstrated in various cellular and animal models. Furthermore, B7-H4 can induce donor-specific tolerance in islet allografts, which holds great promise as an adjunct for modern paradigms of immunosuppression. In the pancreas a relative abundance of B7-H4 in β-cells alludes to novel functions in the pancreatic islets, and ongoing work hints at important roles of endogenous B7-H4 for β-cell health and function. Of note, B7-H4 also displays a unique expression profile unlike that of other B7 family members, and variations in its protein and mRNA splicing species may act as potential biomarkers for T1D. Further research into both the immune-regulatory and β-cell-autonomous roles of B7-H4 promises to elucidate its contributions to β-cell health and survival, thus identifying it as a novel β-cell protective shield for patients suffering from diabetes.

ACKNOWLEDGMENTS

We thank nPOD for providing human pancreas samples from T1D patients, and Ike Barber Human Islet Transplant Lab for human islets.

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P- Reviewer: Liu HK, Pastromas S **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (3): Type 1 diabetes

Why do some patients with type 1 diabetes live so long?

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Received: December 3, 2013 Revised: March 5, 2014
Accepted: April 9, 2014
Published online: June 15, 2014

Abstract

While the lifespan of people with type 1 diabetes has increased progressively since the advent of insulin therapy, these patients still experience premature mortality, primarily from cardiovascular disease (CVD). However, a subgroup of those with type 1 diabetes survives well into old age without significant morbidity. It is the purpose of this review to explore the factors which may help in identifying these patients. It might be expected that hyperglycaemia plays a major role in explaining the increased incidence of CVD and mortality of these individuals. However, while a number of publications have associated poor long term glycaemic control with an increase in both all-cause mortality and CVD in those with type 1 diabetes, it is apparent that good glycaemic control alone cannot explain why some patients with type 1 diabetes avoid fatal CVD events. Lipid disorders may occur in those with type 1 diabetes, but the occurrence of elevated high-density lipoprotein-cholesterol is positively associated with longevity in this population. Non-renal hypertension, by itself is a significant risk factor for CVD but if adequately treated does not appear to mitigate against longevity. However, the presence of nephropathy is a major risk factor and its absence after 15-20 years of diabetes appears to be a marker of long-term survival. One of the major factors linked with long-term survival is the absence of features of the metabolic syndrome and more specifically the presence of insulin sensitivity. Genetic factors also play a role, with a family history of longevity and an absence of type 2 diabetes and hypertension in the family being important considerations. There is thus a complex interaction between multiple risk factors in determining which patients with type 1 diabetes are likely to live into older age. However, these patients can often be identified clinically based on a combination of factors as outlined above.

Key words: Type 1 diabetes; Prognosis; Survival; Coronary artery disease; Cardiovascular disease; Lipids; Metabolic syndrome

Core tip: People with type 1 diabetes are generally assumed to have a shortened lifespan. This contention is supported by a number of epidemiological studies confirming a trend towards premature death, primarily due to cardiovascular disease. However, a subset of type 1 individuals survives for many years, living for over 50 years or more with type 1 diabetes. This review explores the clinical features that are linked to long-term survival in people with type 1 diabetes, allowing identification of these individuals. Recognising these individuals will aid in assessing prognosis, and treating the identified risk factors could improve survival.

Original sources: Distiller LA. Why do some patients with type 1 diabetes live so long? *World J Diabetes* 2014; 5(3): 282-287 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/282.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.282

INTRODUCTION

Prior to the discovery of insulin, patients with type 1 diabetes had an expected lifespan of less than 3 years[1].

With the advent of modern therapy, survival has increased progressively. However, those with type 1 diabetes remain with an increased incidence of coronary artery disease (CAD) and mortality compared to the general population. By 1991, reported standard mortality rates for those with type 1 diabetes under the age of 60 years were 9.1 for males and 13.5 for females[2]. Subsequently, a cohort of 23751 patients from the United Kingdom and diagnosed with diabetes under the age of 30 years between 1972 and 1993 were analysed for cardiovascular mortality up to 2000[3]. These results confirmed higher mortality rates at younger ages for those with type 1 diabetes (Figure 1). Of interest, not only are the mortality rates for women with diabetes considerably higher than for women without diabetes, but also higher than for men without diabetes. Soedamah-Muthu *et al*[4], utilizing the United Kingdom General Practice research database, have also confirmed that the risk of cardiovascular disease (CVD) remains high in patients with type 1 diabetes. Typically, patients with type 1 diabetes reach a 10-year risk of fatal CVD of 5% about 10 to 15 years before the general population. Furthermore, incidence rates of CAD in type 1 patients range between 1.2% and 2% per year, *vs* 0.1% and 0.5% in the general population[5]. The incidence of stroke is also increased in type 1 diabetes, with overall standardised incidence ratios being 17.94 for men and 26.11 for women[6].

It is therefore clear, that despite a better understanding and treatment of appropriate risk factors and better general care, those with type 1 diabetes still have a tendency towards a shortened life span, primarily due to premature CVD. Yet a subgroup of individuals with type 1 diabetes survives well into old age in relatively good health. This review explores the factors that may help to identify these patients. This can be done either by identifying a group of long-surviving type 1 patients and analysing any unique clinical or biological features that may be specific to this cohort, or by assessing surrogate endpoints of vascular disease, such as carotid artery Intima-Media Thickness (IMT) measurement or arterial calcification and identifying those who appear to be "protected" from vascular disease.

THE ROLE OF GLYCAEMIC CONTROL

Type 1 diabetes is a condition of "pure" hyperglycaemia. The only abnormality is one of β-cell failure and insulin deficiency in an otherwise "normal" or "healthy" individual. It could therefore be expected that hyperglycaemia might play a major role in explaining the increased incidence of CVD and mortality seen in these individuals. A number of publications have associated poor long-term glycaemic control with an increase in both all-cause mortality and CVD in those with type 1 diabetes. Grauslund *et al*[7] demonstrated a direct relationship between HbA1c and survival. When patients were categorized into quartiles of HbA1c measurements, patients in the highest quartile had a significantly higher risk of all-cause mortality, cardiovascular mortality and ischaemic heart disease

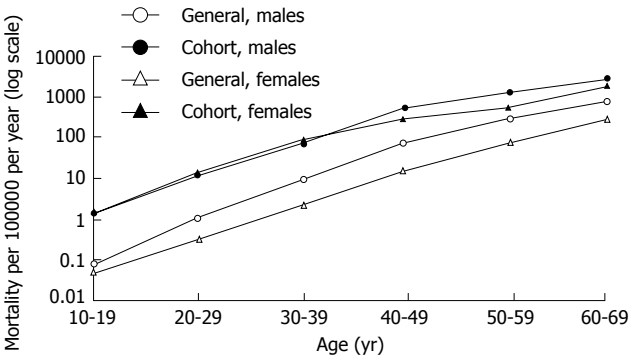

Figure 1 Ischaemic heart disease mortality rates in people with type 1 diabetes *vs* general population (from: Laing *et al*[3]).

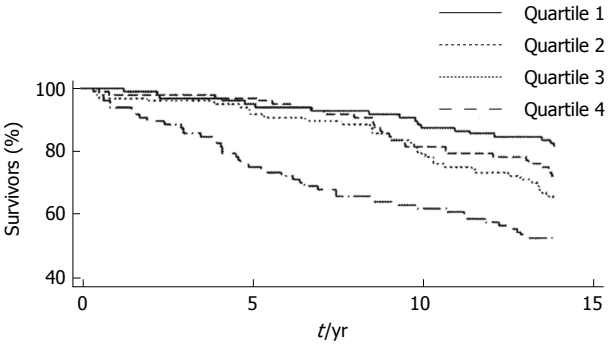

Figure 2 All-cause mortality and the association of glycaemic control (from: Grauslund *et al*[7]).

when compared to patients in the lowest quartile (Figure 2). While at the conclusion of the Diabetes Control and Complications Trial (DCCT) there was no significant difference between the conventional and intensive treatment groups regarding cardiovascular outcomes or death from CVD, the 10-year Epidemiology of Diabetes Interventions and Complications (EDIC) follow-up demonstrated a significant difference between the two groups with regard to both CV outcomes and death. An overall 42% risk reduction was seen in the previously intensively treated group[8]. This sustained effect of improved control in the DCCT years was ascribed to "metabolic memory". Patients followed in the DCCT/EDIC cohort were also submitted to IMT measurements, and it was demonstrated that intensive therapy during the DCCT resulted in decreased progression of IMT six year after the end of the trial[9]. These findings imply that early glycaemic control is an important factor in preventing CVD in type 1 diabetes.

However, good blood glucose levels alone cannot explain why some patients with type 1 diabetes avoid fatal CVD events. In the "Golden Years Cohort" of 400 type 1 patients who survived for over 50 years with diabetes[10], the mean HbA1c was 7.6% (± 1.4), with some of these patients having HbA1c levels as high as 8.5%-9%. None had an HbA1c below 7%. In addition, a number of other publications have shown only a weak correlation between long-term glycaemic control, CVD and mortality. Larsen *et al*[11], performed coronary angiography on 29 asymptomatic patients with a mean duration

of type 1 diabetes of 30.6 years. Of these, 34% had significant coronary artery stenosis. While a significant relationship existed between stenosis and glycaemic control (a 6.1% increase in vessel stenosis for every 1% increase in HbA1c over 18 years), glycaemic control was less significant as a risk factor than the age of the subjects and the effect of elevated serum cholesterol. In another cohort of 125 patients with a mean duration of diabetes of 22 years[12], IMT was compared to an index of lifetime glycaemic exposure. This demonstrated significantly increased IMT only on those at the highest tertile of glycaemic exposure. IMT measurements performed in 148 long-surviving patients with type 1 diabetes (duration > 15 years)[13] showed no significant correlation between HbA1c and IMT, although ordinal logistic regression showed that for every 1% increase in HbA1c, there was a 27% less chance of the IMT falling into the low-risk group (defined as an IMT below 0.6 mm and no plaque). A prospective observational study of a meta-analysis of the relationship between CVD and glycaemic control[14], revealed an only moderate increase in cardiovascular risk with increasing levels of glycated haemoglobin in persons with diabetes mellitus. However, this meta-analysis included patients with both type 1 and type 2 diabetes. The data suggested that there is an increased risk of CVD of 15% for every 1% increase in HbA1c (RR = 1.15; 95%CI: 0.92-1.43).

The evidence therefore suggests that while early good glycaemic control is important in the prevention of CVD and survival, the importance of glycaemic control may diminish as patients survive longer. While glycaemic control is clearly a risk factor for CAD and mortality in type 1 diabetes, this is not the major determinant of survival. Good glycaemic control alone cannot explain why some type 1 patients survive into old age.

LIPIDS IN TYPE 1 DIABETES

Patients with type 1 diabetes may show quantitative lipid disorders. There is a clear relationship between the level of glycaemic control and lipid abnormalities, with an independent correlation between HbA1c and low-density lipoprotein (LDL)-cholesterol, non-high-density lipoprotein (HDL) cholesterol and triglycerides[15]. Abnormal lipid levels are associated with worse cardiovascular outcomes[5]. The lipid profiles of patients with well-controlled type 1 diabetes are very different from those with poor glycaemic control[16], related possibly to the presence of adequate peripheral insulin levels in the better controlled subjects. There are direct metabolic consequences of administering insulin subcutaneously. Peripheral hyperinsulinemia is associated with increased lipoprotein lipase activity[17], which may account for reduced triglyceride levels. In addition, LDL-cholesterol may also be slightly reduced due to decreased very LDL production[18]. The more sensitive the individual is to insulin, the greater is this effect.

As might be expected, Serum LDL-cholesterol and non-HDL-cholesterol levels are positively associated with not only an increase in IMT[9], increased Arterial Stiffness[19] and coronary artery stenosis[11], but also CAD and mortality[5,7,20]. A major factor that appears to be associated with prolonged survival in patients with type 1 diabetes is elevated HDL-cholesterol. HDL levels are often elevated in those with type 1 diabetes. This is more marked with better glycaemic control and may be due to an elevated lipoprotein lipase/hepatic lipase ratio (Increased peripheral lipoprotein lipase activity due to peripheral hyperinsulinemia from subcutaneous insulin administration and normal hepatic lipase activity). Bain *et al*[10] reported a high mean HDL-level in those surviving over 50 years with diabetes (1.84 ± 0.057 mmol/L), and this was associated with lower triglyceride levels (1.49 ± 0.79 mmol/L). In long-surviving type 1 patients, IMT measurements showed a significant inverse association to HDL levels and computed tomography/HDL ratios for all measure of risk (IM thickness and/or plaque)[13]. A number of other studies have supported the protective effects of HDL-cholesterol with regard to CVD[5,7,9,11,20]. In addition to this direct association between HDL-cholesterol and CVD, higher HDL-cholesterol levels may provide protection against the development of albuminuria[21].

Therefore, it can be concluded that in addition to the expected effect of dyslipidaemia (high LDL and non-HDL-cholesterol), HDL-cholesterol itself exerts a significant protective effect on the development of CVD in patients with type 1 diabetes and elevated HDL-cholesterol levels appears to play a major role in longevity in these patients.

BLOOD PRESSURE AS A RISK FACTOR

Hypertension in those with type 1 diabetes is often a manifestation of underlying nephropathy. However, hypertension can also occur as a stand-alone risk factor (non-renal hypertension). A significant positive association between high blood pressure and arterial stiffness in youth with type 1 diabetes was demonstrated in the SEARCH CVD Study[19].

In type 1 diabetes, hypertension without nephropathy has been shown to be a major risk factor for the development of carotid artery plaque [OR = 5.26 ($P < 0.004$)], but the effect of hypertension on IMT was moderate and not significant[13]. In the DCCT/EDIC at 6 years, the presence of hypertension and particularly systolic hypertension was significant, but had less of an effect on IMT than did smoking, lipids or glycaemic control[9]. In the Golden years cohort[10], 29% of the patients were receiving antihypertensive treatment but had nevertheless survived for over 50 years with diabetes.

It therefore appears as though hypertension itself, while a significant risk factor for CVD, if treated does not mitigate against longevity in this population.

MICROVASCULAR DISEASE AS A MARKER OF SURVIVAL

The presence of diabetic nephropathy, microalbuminuria

Table 1 Cox proportional hazard models for risk of cardiovascular disease from nephropathy (from: Grausland *et al*[7])

	All cause mortality	Cardiovascular mortality	IHD
Creatinine > 120 μmol/L	5.1	6.29	4.25
Microalbuminuria	1.32	1.44	1.40
Macroalbuminuria	2.4	2.57	1.77

IHD: Ischaemic heart disease.

or macroalbuminuria is a significant risk factor for CAD, cardiovascular mortality and all cause mortality, and there is a strong independent relationship between albuminuria and CAD (Table 1)[7]. The occurrence of stroke in subjects with type 1 diabetes is also increased by the presence of nephropathy [microalbuminuria: HR = 3.2 (1.9-5.6), macroalbuminuria: HR = 4.9 (2.9-8.2), End Stage Renal Disease: HR = 7.5 (4.2-13.3)][22]. The DCCT/EDIC Study showed a sustained effect of good glycaemic control[23] on the reduction in albumin excretion 7 years after the conclusion of the DCCT study, with an 83% risk reduction in those patients initially treated with intensive therapy, confirming the concept of "metabolic memory". The long-term risk of a reduction in estimated glomerular filtration rate (eGFR) was also shown to be 50% lower among those who were treated early in the course of type 1 diabetes with intensive diabetes therapy than among those treated with conventional diabetes therapy[24]. The development of hypertension was also delayed in the intensively treated group. These effects appeared to be largely mediated by the levels of glycaemia achieved during the DCCT. However, as pointed out by the authors, a long time elapsed between treatment intensification during the DCCT early in the course of the diabetes and the effect on eGFR, and the advantages of improved glycaemic control in persons already with advanced complications may not apply. This further supports the contention that good glycaemic control in the early years of the diabetes may be more important achieved in those who have had the condition for some years.

In type 1 diabetes, the peak incidence of nephropathy occurs between 15 and 20 years after the development of the diabetes[25,26]. Progression from microalbuminuria to overt neuropathy has been shown to reduce from 45% in those with diabetes of less than 15 years, to 26% in those with diabetes of over 15 years duration. By the time someone has had diabetes for over 40 years, it drops to just 4% per year[25]. In this regard, none of the long surviving patient in the "Golden Years cohort"[10] had evidence of overt nephropathy.

It is therefore apparent, that those individuals with type 1 diabetes who are likely to survive, would remain free of any evidence of nephropathy.

No prospective studies in type 1 patients have found a strong independent relationship between retinopathy and CVD or mortality. However, the presence of retinopathy increases the risk of stroke[22]. Severe diabetic retinopathy was common in the "Golden Years Cohort"[10]. Forty-three percent of subjects had had laser therapy and 2% were blind. In relatively long-surviving people with type 1 diabetes, the presence of retinopathy had a significant association with the presence of plaque (OR = 3.65; $P < 0.033$), independent of glycaemic control[13]. However, there was no association between the presence of retinopathy and IMT measurements. It therefore appears as though retinopathy is not a major risk factor for CVD or mortality in those with type 1 diabetes, as opposed to those with type 2 diabetes where the presence of retinopathy may indicate CAD and mortality risk[27].

With regard to peripheral neuropathy, no prospective trials link the presence of neuropathy to either CAD or mortality other than the EURODIAB study, which did detect peripheral and autonomic neuropathy as risk markers for future mortality[20].

TYPE 1 DIABETES AND THE METABOLIC SYNDROME

There is no reason to expect patients with type 1 diabetes to have a lower prevalence of obesity and the metabolic syndrome (MetS) than the general population and a MetS frequency in type 1 patients of over 30% has been reported[28]. A significant relationship exists between mortality and central obesity in those with type 1 diabetes[20] and type 1 subjects with the MetS have been shown to have an increased prevalence of macrovascular disease[29]. The presence of MetS features in patients with type 1 diabetes is associated with risk factors similar to many patients with type 2 diabetes, and the superimposition of the insulin resistance due to obesity or the MetS in a patient who already has type 1 diabetes has been termed "Double diabetes"[30].

Identifying patients with the MetS in the presence of type 1 diabetes is difficult. Of the diagnostic criteria, the presence of dysglycaemia is a foregone conclusion and cannot be used. Hypertension should only be included if it is non-renal as nephropathy-induced hypertension has other implications as outlined above. Quantifying insulin resistance is also difficult and requires a euglycaemic clamp study to document it properly. A derived estimate of glucose disposal rate has been suggested to measure of insulin resistance[31] but this includes the presence of hypertension and waist-hip ratio in the formula and therefore cannot be used in assessing insulin resistance in the context of the MetS, since both of these variables are separate components of the MetS in their own right. Insulin dosage provides a surrogate measurement of insulin resistance in these patients, and in their series of long-surviving type 1 patients, Distiller *et al*[32] arbitrarily chose insulin doses in the top quartile of their series of patients (0.75 U/kg body weight), to be a measure of insulin resistance. In this series, a multiple linear regression analysis showed a significant relationship between waist circumference and insulin dose and carotid artery IMT when corrected for age of onset, current age and duration of diabetes. Interestingly, neither body mass index (BMI) nor HbA1c were significantly associated with carotid artery IMT. Overall, there was a significant

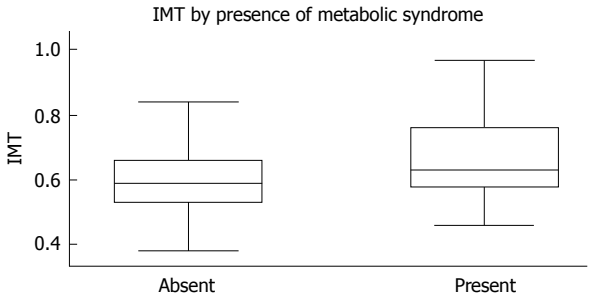

Figure 3 A significant increase in Intima-Media Thickness is seen in patients s with the metabolic syndrome (*P* = 0.003) (adapted from: Distiller *et al*[32]).

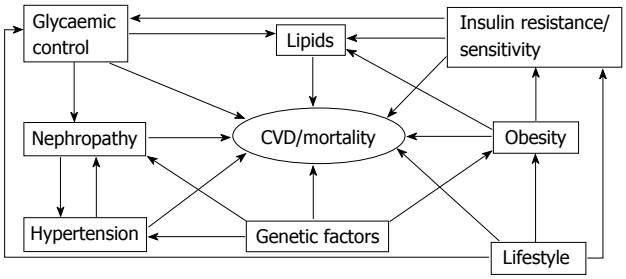

Figure 4 Complex interactions exist between multiple risk factors in determining the outcome for patients with type 1 diabetes. CVD: Cardiovascular disease.

increase in IMT in type 1 subjects with the MetS (Figure 3). A significant association was demonstrated between IMT risk and the number of features of the MetS (P = 0.01). Fifty percent of patients with 0-1 features had low risk IMT, whereas 60% of patients with 3-4 features had high risk IMT measures. This finding was confirmed by the SEARCH CVD Study[16], a longitudinal study of 298 youth with diabetes, where those with the MetS had consistently increased arterial wall stiffness when compared to type 1 patients without the Syndrome and with the same duration of diabetes. This was born out by the "Golden Years Cohort"[10], where the patients were generally on low doses of insulin. The mean daily insulin dose was 37.5 U (± 16.2) (0.52 U/kg body weight), the mean BMI of these long surviving patients was 25 kg/m^2, and HDL-cholesterol was high and triglycerides were low. These features could be considered the antithesis of the MetS.

GENETIC FACTORS

The best predictor of old age is the age one's parents achieved. This adage was supported by the "Golden Years Cohort"[10], where on average, both parents of those surviving 50 years with diabetes lived to over 70 years. Furthermore, a family history of either type 2 diabetes or hypertension has been shown to result in significantly increased IMT in type 1 diabetes subjects[12].

Clearly, a complex interaction exists between multiple risk factors in determining which patients with type 1 diabetes are likely to live into older age (Figure 4). However, these patients can often be identified clinically based on a combination of factors (Table 2).

Table 2 Identifying features of long-surviving patients with type 1 diabetes

Reasonable (not necessarily ideal) glycaemic control
High HDL-cholesterol levels
Low daily insulin requirements ("insulin sensitive")
Normal body weight
Non-smokers
Lower blood pressures
Microalbumin negative after 15-20 yr of diabetes
Family history of longevity

HDL: High density lipoprotein.

CONCLUSION

While the longevity of those with type 1 diabetes has improved considerably over the past century, these patients remain with a reduced life expectancy compared to the non-diabetic population. Nevertheless, a subgroup of these individuals may survive into older age despite their diabetes. Certain clinical and biochemical features can identify these people. This understanding may provide clinicians with further evidence that correction of modifiable risk factors like glycaemic control, blood pressure control, avoidance of excessive weight gain and lipid control is vital in ensuring the ongoing longevity of patients with type 1 diabetes.

ACKNOWLEDGMENTS

I would like to thank Michael Brown for his invaluable assistance in editing and preparing this manuscript.

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P- Reviewers: Masuo K, PIzawa K **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

12q24 locus association with type 1 diabetes: *SH2B3* or *ATXN2*?

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Received: October 29, 2013 Revised: March 13, 2014
Accepted: April 11, 2014
Published online: June 15, 2014

Abstract

Genetic linkage analyses, genome-wide association studies of single nucleotide polymorphisms, copy number variation surveys, and mutation screenings found the human chromosomal 12q24 locus, with the genes *SH2B3* and *ATXN2* in its core, to be associated with an exceptionally wide spectrum of disease susceptibilities. Hematopoietic traits of red and white blood cells (like erythrocytosis and myeloproliferative disease), autoimmune disorders (like type 1 diabetes, coeliac disease, juvenile idiopathic arthritis, rheumatoid arthritis, thrombotic antiphospholipid syndrome, lupus erythematosus, multiple sclerosis, hypothyroidism and vitiligo), also vascular pathology (like kidney glomerular filtration rate deficits, serum urate levels, plasma beta-2-microglobulin levels, retinal microcirculation problems, diastolic and systolic blood pressure and hypertension, cardiovascular infarction), furthermore obesity, neurodegenerative conditions (like the polyglutamine-expansion disorder spinocerebellar ataxia type 2, Parkinson's disease, the motor-neuron disease amyotrophic lateral sclerosis, and progressive supranuclear palsy), and finally longevity were reported. Now it is important to clarify, in which ways the loss or gain of function of the locally encoded proteins SH2B3/LNK and ataxin-2, respectively, contribute to these polygenic health problems. SH2B3/LNK is known to repress the JAK2/ABL1 dependent proliferation of white blood cells. Its null mutations in human and mouse are triggers of autoimmune traits and leukemia (acute lymphoblastic leukemia or chronic myeloid leukemia-like), while missense mutations were found in erythrocytosis-1 patients. Ataxin-2 is known to act on RNA-processing and trophic receptor internalization. While its polyglutamine-expansion mediated gain-of-function causes neuronal atrophy in human and mouse, its deletion leads to obesity and insulin resistance in mice. Thus, it is conceivable that the polygenic pathogenesis of type 1 diabetes is enhanced by an SH2B3-dysregulation-mediated predisposition to autoimmune diseases that conspires with an ATXN2-deficiency-mediated predisposition to lipid and glucose metabolism pathology.

Key words: Diabetes mellitus type 1; 12q24; *ATXN2*; Obesity; *SH2B3*; Autoimmune

Core tip: Within the multifactorial pathogenesis of type 1 diabetes mellitus (T1D), a genetic risk mediated by the chromosome 12q24 locus was consistently observed. Mutations in the *ATXN2* gene there trigger the pathogenesis of obesity, while mutations in the SH2B3 gene there trigger the pathogenesis of autoimmune processes. Given that both genes show co-regulated expression, their combined effects may drive these two core aspects of T1D. Tissue and phenotype studies of mouse mutants will identify molecular targets for causal therapies.

Original sources: Auburger G, Gispert S, Lahut S, Ömür Ö, Damrath E, Heck M, Başak N. 12q24 locus association with type 1

diabetes: *SH2B3* or *ATXN2*? *World J Diabetes* 2014; 5(3): 316-327
Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/316.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.316

INTRODUCTION

The pathogenesis of many common multifactorial diseases was successfully elucidated over the past years, principally through genome-wide association studies (GWAS) in many thousands of sporadic patients *vs* control individuals. For diabetes mellitus type 1 (T1D), more than 40 chromosomal loci were uncovered to modulate disease risk[1,2]. However, now the challenge consists in establishing causality between one of the multiple genes contained in any locus and one of the disease features. One promising approach is the careful consideration of phenotypes and pathology caused by disruption or overexpression of any candidate gene, *e.g.*, in mouse, and the subsequent comparison with relevant traits that occur within the first years of the disease course. Thus, clinical information may help to guide the characterization of mutant animals, while conversely the tissue analysis of mutant animals may help to elucidate presymptomatic stages of disease. A particularly complex example is the subject of this review-the association of T1D and many other medical conditions with mostly two single nucleotide polymorphisms (SNPs) on chromosome 12q24-rs3184504 and rs653178.

THE EXCEPTIONALLY PLEIOTROPIC DISEASE SUSCEPTIBILITY LOCUS ON CHROMOSOME 12Q24 EXTENDS FROM THE *SH2B3* GENE ACROSS THE *ATXN2* GENE, BUT MAY STRETCH BEYOND THESE BORDERS

Chromosome 12q contains one of the largest blocks of linkage disequilibrium (LD) in the human genome[3]. It was observed early on in European/Asian/African populations and found to span > 1 Megabase pairs (Mbp) across several genes including the growth repressor *SH2B3*, the RNA processing factor *ATXN2*, the nuclear localization inhibitor *BRAP*, the mitochondrial fatty acid beta-oxidation enzyme *ACAD10*, the alcohol metabolism enzyme *ALDH2*, and the stress kinase *MAPKAPK5*[4]. The core LD block was localized to exon 1 of the *ATXN2* gene in a population of European ancestry, and was explained by positive selection of the (CAG)-repeat size in this exon[4]. Indeed, the most frequently observed disease associations at this 12q24 locus are within a 200000 basepairs (bp) fragment, which comprises the *ATXN2* gene and the immediately adjacent *SH2B3* gene (Figure 1). According to the United States National Center for Biotechnology Information reference sequences, human *SH2B3* is transcribed in orientation from the centromere, covering about 46000 bp, and spans 9 predicted exons to constitute an mRNA of 5425 nucleotides, which encodes a protein of 575 amino acids. *ATXN2* is transcribed in orientation from the telomere, covering about 147000 bp, and spans 24 predicted exons with several splice-isoforms, of which the longest constitutes an mRNA of 4712 nucleotides and encodes a protein of 1313 amino acids. The missense SNP rs3184504 in *SH2B3* open reading frame (resulting in the substitution W262R) was observed in perfect cosegregation ($r^2 = 1$) with the SNP rs653178 deep within intron 2 of the *ATXN2* gene[5], in spite of a physical distance of 123148 bp. Since rs653178 is far away from *ATXN2* splice sites and since the W262 codon in SH2B3 is not conserved between human and mouse[6], both of these polymorphisms are probably innocent bystanders and are noticed only through their frequency, depending on their random distribution within population stratifications. They are presumably coinherited with other rare sequence variants, *e.g.*, within the promoters or within the mRNA 3'-untranslated regions, which alter the transcript expression levels slightly upwards or downwards. Indeed, both of these cosegregating *SH2B3* and *ATXN2* variants correlated with significant changes in the expression of both *ATXN2* and *SH2B3* mRNAs[7]. This coinheritance together with correlated expression changes makes it inherently difficult to establish causality between any of the individual traits within a complex disease and any of the neighbouring genes. This is exemplified by the allocation of six hematologic and three blood pressure traits to the region from *SH2B3* to *ATXN2* by genome-wide studies, reflecting the exceptional pleiotropy of this locus[8]. The 12q24 linkage disequilibrium block in some studies of restricted populations included further genes, namely *CUTL2*, *FAM109A*, *SH2B3*, *ATXN2*, *BRAP*, *ACAD10*, *ALDH2*, *MAPKAPK5*, *TMEM116*, *ERP29*[9], *NAA25/C12orf30*, *TRAFD1*, *HECTD4/C12orf51*, *RPL6*, *PTPN11*[10-12], thus extending across 1.5 Mbp. For these reasons it is crucial to consider monogenic mutants for each gene and their phenotypic effects, so as to decide which of them might contribute to each of the diseases. However, for most of these genes the relevant mouse mutants are not yet characterized.

NULL MUTATIONS IN MOUSE AND HUMAN DEMONSTRATE *SH2B3* TO REPRESS THE PROLIFERATION OF WHITE BLOOD CELLS, IN PARTICULAR B-LYMPHOCYTES

The generation of mice with deletion of SH2B3 (also called Lnk) demonstrated primary splenomegaly and extramedullary hematopoiesis with progenitor hypersensitivity to various cytokines[13]. It caused the accumulation of pre-B and immature B-lymphocytes in enlarged spleens as well as an increase in B-lineage cells in the bone marrow, in parallel to unimpaired T-cell de-

SH2B3-ATXN2 genomic locus

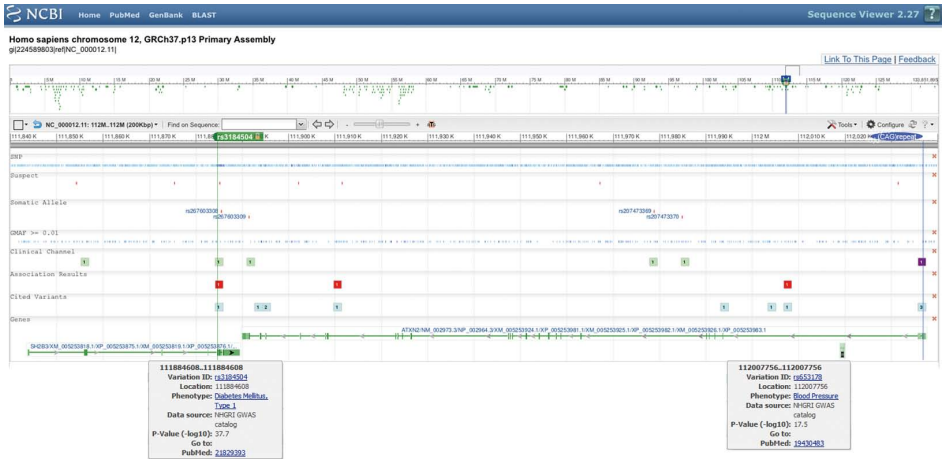

Figure 1 The core 200000 bp region of the chromosome 12q24 locus covering the immediately adjacent *SH2B3* and *ATXN2* genes, with an illustration of the single nucleotide polymorphism rs3184504 encoding the W272R missense variant of the SH2B3/LNK protein (as shown in the United States National Center for Biotechnology Information database) as well as the (CAG)-repeat structure encoding the unstable polyglutamine domain of the ataxin-2 protein.

velopment in thymus[14]. It accelerated and exacerbated oncogenic JAK2-induced myeloproliferative diseases through an expansion of myeloid progenitors, accelerated myelofibrosis and finally features of chronic myeloid leukemia (CML). These murine data supported notions that SH2B3 directly inhibits oncogenic JAK2 and cooperates with the *BCR/ABL* oncogene in the development of CML[15]. Deletion of SH2B3 was also observed in a genomic and transcriptomic study of patients with BCR-ABL1-positive acute lymphoblastic leukemia with poor outcome (Ph-like ALL), together with promising therapeutic benefits from tyrosine kinase inhibitors[16]. Human germline homozygous *SH2B3* mutations including a frameshift with translation stop resulted in growth retardation, high white cell counts in parallel to anemia and thrombocytopenia, splenomegaly and liver cirrhosis, autoimmune Hashimoto thyroiditis, speech delay and ALL. In addition, this study identified homozygous somatic *SH2B3* frameshift mutations in ALL cases[17]. A 5 bp deletion of SH2B3, which was predicted to affect both the PH domain and the SH2 domain, manifested clinically as primary myelofibrosis. In contrast, a somatic *E208Q* missense mutation in the PH domain was observed in a patient with essential thrombocythemia[18]. SH2B3 was also shown to interact with platelet-derived growth factor receptor and repress its downstream signaling[19]. Interestingly, a selective increase in red blood cells (isolated erythrocytosis) was observed in two individuals with the *SH2B3* missense mutations E208X and A215V[20]. However, SH2B3 sequencing in 23 erythrocytosis patients uncovered only one non-synonymous polymorphism of unclear relevance[6]. Systematic SH2B3 sequencing analysis in 42 patients with chronic phase myeloproliferative neoplasms detected a missense mutation in 7% of cases, either in the SH2 domain or in the C-terminal domain, which were always accompanied by a *JAK2* mutation[21]. Myeloproliferative *SH2B3* mutations within the PH domain were also shown to reduce SH2B3 function without altering its binding properties to JAK2, CBL and 14-3-3[22]. An analysis of peripheral mononuclear blood cells stimulated with anti-CD28 and anti-CD3 antibodies detected an increased proliferation of T-lymphocytes in carriers of the W262R missense SH2B3 variant, independent of the presence of juvenile type 1 diabetes[23]. *In vitro* studies had previously shown SH2B3 to attenuate the ability of SH2B1 to promote JAK2 activation and subsequent tyrosine phosphorylation of insulin receptor substrate-1 by JAK2[24]. SH2B3-deficient hematopoietic stem cells displayed an increased postnatal expansion and enhanced thrombopoietin responsiveness[25]. In subsequent studies they showed increased resistance to apoptosis due to enhanced expression of Bcl-xL upon thrombopoietin stimulation[26]. A limitation of growth by SH2B3 was also observed in the rat neuronal PC12 cell line and in primary cortical neurons, where neurotrophin-induced neurite outgrowth was downregulated by the binding of SH2B3 to the phosphorylated neurotrophin receptor TrkA and the repression of downstream signaling[27].

AUTOIMMUNE DISEASES (EOSINOPHIL NUMBERS, COELIAC DISEASE, JUVENILE IDIOPATHIC ARTHRITIS, RHEUMATOID ARTHRITIS, THROMBOTIC ANTIPHOSPHOLIPID SYNDROME, LUPUS ERYTHEMATOSUS, MULTIPLE SCLEROSIS, HYPOTHYROIDISM, VITILIGO) MAY BE MODULATED BY SH2B3

Possibly as an effect of SH2B3 on B-lymphocyte proliferation, the 12q24 locus modulates the risk for various autoimmune diseases. A GWAS in the Icelandic popula-

tion studying eosinophil counts observed association with the *SH2B3* SNP rs3184504[28]. A GWAS into coeliac disease found the *SH2B3* SNP rs3184504 and the *ATXN2* intronic SNP rs653178 to be associated[29]. Follow up studies of coeliac disease focusing on 9 and 11 candidate SNPs confirmed the association with *SH2B3*[30,31], and reported upregulation of *SH2B3* mRNA expression levels in intestinal mucosa to be triggered by coeliac disease and by the risk allele T of the *SH2B3* SNP rs3184504[31]. Further haplotype studies were confirmatory, and functional experiments indicated that carriers of the rs3184504 risk allele show stronger activation of the NOD2 recognition pathway in response to lipopolysaccharides and muramyl dipeptide[32]. A candidate study of sixteen SNPs known from coeliac disease and from T1D found an association of the *ATXN2* SNP rs653178 with juvenile idiopathic arthritis[33]. GWAS studies into rheumatoid arthritis indicated association with *SH2B3* particularly among rheumatoid-factor-positive patients[34]. A GWAS meta-analysis confirmed that the *ATXN2* intronic SNP rs653178 is associated not only with coeliac disease, but also with rheumatoid arthritis[35]. A study of thrombophilia in antiphospholipid antibody positive individuals by array-comparative genomic hybridization analysis of copy number variations with subsequent fine mapping identified a risk haplotype comprising one *SH2B3* SNP and two *ATXN2* SNPs[36]. A GWAS of systemic lupus erythematosus observed association with the SNP rs17696736 within the *ERP29* gene downstream from *SH2B3*[9]. A candidate study of 12 SNPs in almost 3000 Spanish multiple sclerosis patients detected association with the *SH2B3* SNP rs3184504[37]. A GWAS into hypothyroidism reported the *SH2B3* SNP rs3184504 to be associated, with autoimmune Hashimoto thyroiditis as a likely explanation for this observation[38]. A GWAS into the autoimmune skin disease vitiligo reported an association with the 12q24 locus extending from the *SH2B3* across the *ATXN2* gene[39].

T1D MELLITUS

The first GWAS into T1D encountered a maximal association with the 12q24 SNP rs17696736 in an intron of the *C12ORF30/NAA25* gene, while the effect was consistently observed also in its neighbourhood across a 1.5 Mbp LD block[10]. An extended GWAS confirmed this observation and pointed out that the association with the W272R missense variant encoded in exon 3 of SH2B3 was sufficient to model the regional effect[40]. GWAS of additional cases corroborated the association with *SH2B3*[41], a further GWAS with meta-analysis and combined comparisons supported the association with rs3184504[42], and also a GWAS of affected sib-pair families showed association with the region from the *SH2B3* SNP rs739496 across the *ERP29* SNP rs17696736 until the SNP rs10850061 beyond PTPN11[11,43]. GWAS of autoantibody positive T1D patients again detected the association with SH2B3[44,45]. GWAS of soluble intercellular adhesion molecule-1 levels as an endothelium-derived inflammatory biomarker in diabetes and infarction also showed the association with the *SH2B3* SNP rs3184504[46]. Candidate studies of 2 and 21 SNPs in T1D cases from Russia and United States, respectively, replicated the SH2B3 association[47,48]. Since the effect is so consistent, *SH2B3* SNP genotyping was integrated into a signature of 8 polymorphisms that provide optimal prediction of T1D risk[49]. However, it is likely that the *SH2B3* sequence variant rs3184504 is not biologically responsible by itself, since sequencing studies failed to find similar SH2B3 variants in NOD mice that model many T1D features[50].

EVIDENCE FROM MOUSE MUTANTS IMPLICATES *ATXN2* IN METABOLIC SYNDROME

While the autoimmune component of T1D might be explained by the SH2B3 effect on lymphocyte proliferation, some metabolic features of T1D might be exacerbated by the ataxin-2 effect on glucose and lipid metabolism. Mice with targeted deletion of Atxn2 exon 1 and frameshift in homozygous state displayed marked obesity and infertility in two independently generated mutant lines[51,52]. Hepatic lipid and glycogen accumulation was evident already at age 6 mo. As in other insulin resistance syndromes, pancreatic and blood serum insulin levels were increased, in parallel to a reduction of insulin receptor (IR) protein levels in the liver, in spite of increased IR mRNA levels. Serum cholesterol was significantly increased[52]. Although ataxin-2 is mostly localized at the rough endoplasmic reticulum and has strong effects on mRNA processing[53-59], its effect on the IR is possibly explained through interactions with the endocytic internalization machinery of receptor tyrosine kinases[60,61]. TDP-43 is an interactor protein of ataxin-2 *via* joint RNA-binding[57], was also demonstrated to regulate glucose homeostasis and fat deposition, with its levels showing direct correlation with the expression levels of the obesity gene *Tbc1d1*, while its deletion affects the splicing of apolipoprotein A-Ⅱ[62-64].

EVIDENCE FROM HUMAN MUTATIONS IMPLICATES *ATXN2* IN OBESITY

The investigation of obesity in 92 children by systematic sequencing of the *ATXN2* coding regions demonstrated a greatly increased frequency of the SNP rs695872 allele C and an overrepresentation of (CAG)-repeat sizes > 22[65]. Indeed, obesity and polyphagia were marked features of infants in middle stages of the neurodegenerative process caused by (CAG)-repeat expansions in ATXN2[66]. Thus, monogenic evidence links obesity to ATXN2 both in mice and in human. This is possibly reflected by a genome-wide SNP genotyping analysis, where SH2B3 variants were associated with low-density lipoprotein (LDL) cholesterol[67]. Interestingly, an association with obesity was also observed for the ataxin-2

binding protein 1 (A2BP1 or RBFOX1) both in a GWAS among Pima Indians and in a candidate approach among French Caucasian adults[68].

ATXN2 IS IMPORTANT FOR NEURODEGENERATIVE DISEASES

The polyglutamine (polyQ) domain at the N-terminal end of ataxin-2 normally has a size of Q22-23, usually encoded by a (CAG)$_8$CAA(CAG)$_4$CAA(CAG)$_8$ sequence in exon 1 of the *ATXN2* gene on chromosome 12q24. Its unstable expansion to large sizes beyond (CAG)$_{31}$ is the monogenic cause of an autosomal dominant multisystem atrophy of the nervous system, which was named spinocerebellar ataxia type 2[69-86]. CAG-repeat expansions with cytosine adenosine adenosine (CAA) interruptions may also manifest as Parkinson's disease[87,88]. Intermediate CAG-repeat sizes of 26-31 units, sometimes with CAA interruptions, act as polygenic risk factor for the motor-neuron disease amyotrophic lateral sclerosis[57,89]. Intermediate CAG-repeat expansions enhance also the risk for progressive supranuclear palsy[90]. Published evidence suggests that the polyglutamine expansions increase the half-life of ataxin-2 and that a gain-of-toxic-function through accumulation of ataxin-2 aggregates with sequestration of interactor proteins such as the poly(A)-binding-protein PABPC1 underlies the neurodegenerative process[57,91]. In spite of the vast evidence that excess ataxin-2 is the biological cause for neuronal death, SNP genotyping and association studies curiously found an *SH2B3* allele haplotype to be more informative and to better predict amyotrophic lateral sclerosis risk than the ATXN2 alleles[92]. This observation underscores old experiences that maximal linkage logarithm of odds scores and maximal haplotype association scores within any chromosomal region depend on random population stratification effects and on the frequency/informativity of alleles. Thus, they are not suitable for the fine mapping of disease genes.

LONGEVITY

Interestingly, the discovery set of a GWAS of exceptional longevity in centenarians detected a significant association with the *ATXN2* SNP rs653178, in parallel to several other disease associated SNPs, while the strongest effect correlated with the SNP rs2075650 at the *TOMM40*/apolipoprotein E (*APOEO* locus. TOMM40 encodes the channel forming subunit of the translocase across the mitochondrial outer membrane, while APOE encodes the apolipoprotein E, which mediates the binding and clearance of lipoprotein particles such as chylomicrons and very LDLs. Apolipoprotein E polymorphisms are the main known genetic factors associated with the risk of Alzheimer's disease[93,94]. While it remained unclear in this longevity GWAS, whether an LD effect was consistently observed also for SNPs that surround ATXN2, and whether blood cell traits, autoimmune disorders, obesity, neurodegenerative processes or vascular pathology were underlying this observation, the authors reported their observation of a reduced frequency of the *ATXN2* SNP rs653178 allele T among centenarians [with a log10(BayesFactor) of 1.2] in the light of previous ATXN2 GWAS association data with hypertension[93,94].

KIDNEY DISEASE, MICROCIRCULATION, HYPERTENSION AND CARDIOVASCULAR INFARCTION

Indeed, several independent GWAS found renal function (estimated glomerular filtration rate on the basis of cystatin c) and chronic kidney disease to be modulated by the rs653178 variant within an intron of the *ATXN2* gene in populations of European and African ancestry[5,95-97]. Also a GWAS into plasma levels of beta-2-microglobulin as a biomarker of kidney function, cardiovascular diseases and mortality reported an association with the *ATXN2* SNP rs653178[98]. Furthermore, a recent GWAS into serum urate concentrations uncovered an association with the *ATXN2* SNP rs653178[99]. The analysis of 83 candidate SNPs showed kidney disease variants to be associated with vascular phenotypes only in the case of rs653178 within the *ATXN2* gene and two SNPs at the *SH2B3* locus[100]. A GWAS studying microcirculation as measured by retinal venular caliber reported 4 loci, with only the rs10774625 SNP within an *ATXN2* intron showing also significant association with hypertension and coronary heart disease[12]. The *ATXN2* SNP rs653178 and the *SH2B3* SNP rs3184504 association with diastolic as well as systolic blood pressure, mean arterial pressure and pulse pressure was reported in three independent GWAS of populations with European and African ancestry[7,101-103]. Similarly, an association of the *SH2B3* SNP rs3184504 with diastolic and systolic blood pressure and hypertension was detected in a GWAS of 200000 individuals of European descent[104]. A GWAS association of the *ATXN2* SNP rs653178 with myocardial infarction was shown in Icelandic individuals[28]. A recent candidate SNP study replicated the association between the *SH2B3* SNP rs3184504 and coronary heart disease also in South Asian patients[105]. Thus, it appears that the 12q24 locus has a marked effect on vascular pathology.

RED BLOOD CELL TRAITS

It is unclear whether the above vascular disorders are consequences of vessel wall pathology or of blood cell pathology. It may therefore be relevant that a GWAS into the genetic basis of six traits of erythrocytes (including hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and red blood cell count) also showed associations with the 12q24 locus from *SH2B3* across the *ATXN2* gene[106].

CONCLUSION

For further mechanistic insights it will be important to

generate and characterize rodent mutants for each of the genes in the pleiotropic 12q24 disease susceptibility locus.

With the limited knowledge available so far, it is credible that SH2B3 modulates B-lymphocyte proliferation and autoimmune traits. Ataxin-2 gain-of-function is a well-established modulator of several neurodegenerative diseases, while its deficiency appears to predispose to insulin resistance, blood cholesterol elevation, hepatic glycogen and lipid accumulation with overall obesity. Thus, downstream effects of both genes might cooperate to enhance the risk for type 1 diabetes.

Since T1D is an age-associated disease, it will be important to age Atxn2-null mice beyond 6 mo to the end of their natural lifespan around 2 years. This will allow us to assess whether their obesity leads to hypertension and vascular pathology, *e.g.*, in kidneys, whether red blood cell traits are altered, and whether their longevity is abnormal. In particular, the insulin resistance/obesity/dyslipidemia/hepatosteatosis induced by *Atxn2*-null mutations should be studied regarding their long-term consequences. Mechanistically, it will be intriguing to elucidate how the RNA processing effects of ataxin-2 lead to this pathology.

In view of the polyQ expansion effects extending the protein half-life and causing a gain-of-function of ataxin-2, it is conceivable that the polyQ shrinkage sizes (Q13-21) could mediate a decreased half-life of the protein and a partial loss-of-function. Thus, these rare variants might be associated with phenotypes that were observed in the Atxn2-null mouse, such as obesity, insulin-resistance and diabetes mellitus.

ACKNOWLEDGMENTS

We are grateful to Ulrich Müller and Jan Rozman for critical reading of the manuscript.

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P- Reviewer: Joseph P **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (3): Type 1 diabetes

Prognostic value of endothelial dysfunction in type 1 diabetes mellitus

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Received: June 3, 2014 Revised: June 30, 2014
Accepted: July 18, 2014
Published online: October 15, 2014

Abstract

Patients with diabetes mellitus are at high risk of developing atherosclerosis, associated with higher rates of micro and macro vascular involvement such as coronary artery disease and renal disease. The role of hyperglycemia to induce synthesis of reactive oxygen species by the oxidation of glucose, leading to an increased production of advanced glycosylation end products, as well as inflammation and oxidative stress has been proposed as a possible mechanism in the pathogenesis of endothelial dysfunction (ED). The interaction between C-peptide - the connecting segment of pro-insulin-and nitric oxide in vasodilation is also discussed. Therefore, endothelial dysfunction has been identified as an early marker of vascular disorder in type 1 and type 2 diabetes mellitus. In some other diseases, ED has been considered an independent predictor of vascular disease, regardless of the method used. Studies have demonstrated the importance of endothelial dysfunction as an useful tool for identifying the risk of vascular complications in patients with type 1 diabetes mellitus, particularly as regards to renal impairment. The aim of this review is to clarify the prognostic value of endothelial dysfunction as a marker of vascular disease in these subjects.

Key words: Endothelial dysfunction; Type1 diabetes; Prognostic; Cardiovascular disease; Pathogenesis

Core tip: This review is divided into two parts: first we discuss aspects related to the pathogenesis of endothelial dysfunction in type 1 diabetes mellitus. In the second, are pointed out and critically discussed the scientific evidence about the important role of endothelial dysfunction, independent of the method used for its diagnosis, as an early marker of cardiovascular and renal complications in this population.

Original sources: Ladeia AM, Sampaio RR, Hita MC, Adan LF. Prognostic value of endothelial dysfunction in type 1 diabetes mellitus. *World J Diabetes* 2014; 5(5): 601-605 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/601.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.601

INTRODUCTION

Diabetes mellitus patients have a high risk to develop atherosclerotic disease[1]. The macro- and microvascular complications are the main cause of morbidity and mortality, especially in those with more than five years of disease[2-4]. Endothelial dysfunction (ED) has been identified as an early marker of vascular disease in type 1 diabetes mellitus (T1DM)[5]. In some other conditions, ED has been an independent predictor of cardiovascular risk[6].

This review aims to evaluate the endothelial dysfunc-

tion role as a prognostic factor of vascular complication in patients with T1DM.

PATHOGENESIS OF ENDOTHELIAL DYSFUNCTION IN T1DM

The role of vascular endothelium on the pathogenesis of vascular disease has been better known in the last 30 years. Adequate endothelial function depends on the healthy balance between vasoconstrictor and vasodilator substances that interact in the endovascular environment[7,8]. Nitric oxide (NO), identified by Furchgott *et al*[9], is synthetized from L-arginine by nitric oxide synthase (eNOS) in the presence of oxygen, nicotinamide adenine dinucleotide phosphate and BH4 (tetrahydrobiopterin). This substance produced on endothelial cells diffuses itself into smooth muscle cells and platelets, where it stimulates the activity of the soluble guanylate cyclase and hence production of cyclic GMP promoting, in turn, relaxation of the muscle layer of the vessel and reduces platelet aggregation. On the other hand, NO reduction is associated with increased vascular injury, because it enhances platelet aggregation and increases monocyte adhesion to vascular endothelium; as well it stimulates proliferation of smooth muscle cells[10].

In pathologic situations, as diabetes, numerous mechanisms as: (1) decreased synthesis or inactivation of NO; and (2) increased production and release of vasoconstrictor substances have been proposed to explain the ED. In addition, metabolic changes favoring increased production of free radicals as well as advanced glycosylation end products (AGEs) are able to accelerate the nitric oxide inactivation[10].

Considering that the major metabolic disturbance in diabetes is hyperglycemia, it has been suggested that it may induce the synthesis of reactive oxigen species, by the oxidation of glucose[11], leading to an increased production of AGEs[12], among other mechanisms. On the other hand, a recent study demonstrates that hypoglycemia is also associated with ED, oxidative stress and inflammation. Moreover, worsening of endothelial function was greater in those who went from hypoglycemia to hyperglycemia than those recovered to a state of normoglycemia[13].

Other substances involved in the pathogenesis of endothelial dysfunction in T1DM are insulin and C-peptide. Several studies have shown that the vasodilator effects of insulin depends on the synthesis of nitric oxide, since the use of substances that block eNOS, inhibits the increase of blood flow mediated through the action of insulin[14-16]. Moreover, acute administration of C-peptide-a connecting segment of pro-insulin-is able to increase blood flow in subjects with T1DM after exercise or at rest, but not in normal subjects[17,18]. As well, a prolonged infusion of C-peptide in type 1 diabetic subjects improves kidney function[19] by a mechanism that involves the interaction between nitric oxide activity, and Na^+K^+ATPase[20,21]. So, it is important to understand that the pathogenesis of endothelial dysfunction in T1DM is complex and involves metabolic and hormonal changes; in particular, the role of insulin deficiency that leads to a decreased production of nitric oxide, increased oxidative stress in the vascular milieu with consequent decreased in the ability to promote vessels dilation. Furthermore, it is suggested that a better control of metabolic changes by insulin replacement can decrease the aggression of endothelial cells.

Other aspects of the pathogenesis of vascular abnormalities in diabetic subjects deserve attention. T1DM and T2DM are associated with a reduction in the number of endothelial progenitor cells (EPCs)[22-24]. It is interesting to note that this reduction is related to the severity of peripheral vascular disease which reinforces the importance of EPCs as a marker of vasculopathy in diabetic patients[25]. Moreover, potent vasoconstrictor such as angiotensin II and endothelin promote endothelial dysfunction in the metabolically altered environment of diabetes[26]. This knowledge is relevant since it may allow the emergence of new therapeutic perspectives. It is noteworthy that it has already been demonstrated that oral treatment with bosentan, endothelin receptor antagonist, for 4 wk, improves endothelial function in T2DM[27].

PROGNOSTIC VALUE OF ENDOTHELIAL DYSFUNCTION

The literature clearly suggests that metabolic and hormonal disorders present in T1DM injure the endothelial cells favoring endothelial dysfunction and initiation of the atherogenic process. A longitudinal study published recently suggests that flow-mediated vasodilation is an useful tool to stratify T1DM children according to cardiovascular risk, as well as for the long term follow-up[28]. However, the prognostic value of endothelial dysfunction as a marker of vascular complications should be further analyzed.

A 10-year follow-up prospective cohort study involving young T1DM adults with a mean disease duration of 19 years, evaluated the ability of adhesion molecules in predicting coronary artery disease (CAD) defined by angina, confirmed myocardial infarction, stenosis > 50%, ischemic electrocardiogram, or revascularization. With this purpose, a nested case-control study involving 60 patients who developed CAD and 72 patients without the disease was performed. Dosages of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 and E-selectin were performed from stored samples prior to the cardiovascular event. Although there was a correlation between adhesion molecules and lipid variables, considered an unquestionable cardiovascular risk factor in type 1 diabetes, only E-selectin was an independent predictor of CAD (HR = 1.07, 95%CI: 1.01-1.15, $P < 0.03$)[29].

Another cross-sectional study that included patients with T1DM without cardiovascular disease and a comparison group of healthy subjects, sought to identify association between endothelial dysfunction [flow-mediated vasodilation (FMD)] and subclinical cardiovascular dis-

ease. It was then observed a strong inverse correlation between FMD and systolic dysfunction (r = -0.70, P < 0.0001), diastolic dysfunction (r = -0.77) and duration of T1DM (r = -0.61,) P < 0.0001 for the three variables[30]. The association between ED and other markers of subclinical CAD, as the carotid intima-media thickness (IMT), was evaluated in a study that included T1DM patients and non-diabetic children-without significant differences in weight, age, blood pressure and gender. This study demonstrated that the T1DM group had lower peaks of FMD response and higher IMT when compared to controls (P < 0.001). Moreover, in the multivariate analysis, there was a strong association between increased IMT and decreased FMD in the group of children with diabetes (P < 0.03). However, the data in the literature are still conflicting. A study involving patients with T1DM and healthy subjects showed no difference between the IMT of the two groups, although endothelial function had been worse in T1DM group and correlated with glycemic control[31].

On the other hand, a recent study that evaluated endothelial function, IMT and ventricular function in 30 children and adolescents with T1DM compared with 30 healthy subjects matched by gender, age, and body mass index, found a lower FMD response, increased IMT and impaired diastolic function with lower early peak flow velocity, decreased E/A ratio, increased early filling deceleration time in T1DM patients. Furthermore, these changes were more evident in patients with poor glycemic control[32].

Several studies have shown the importance of endothelial dysfunction as a marker of renal impairment. In 2005, we demonstrated that FMD had an inverse correlation with microalbuminuria (r = -0.50, P = 0.049) in children and adolescents with a short duration diabetes (2.9 + 1.2 years) calling attention to the value of the endothelial dysfunction as a very early marker of vascular complications[4]. This association was also demonstrated in patients with disease duration > 10 years, with the following features: individuals with proteinuria and chronic renal failure (CRF) had FMD 7% and 4% respectively, while those with normal albumin excretion or microalbuminuria showed FMD > 8%, considered the lower limit of normality for flow-mediated vasodilation in adults[33]. In this study, there was a continuous, progressive and significant increase in the levels of endothelin-1 and C-reactive protein in individuals (1) without microalbuminuria; (2) with microalbuminuria; (3) with proteinuria; and (4) CRF. In addition, the sensitivity coefficient to shear stress endothelium was inversely correlated with glomerular filtration rate (GFR) (r = -0.48, P = 0.03). This aspect can be somewhat reinforced by another recent study that demonstrated that pulse pressure was associated with a decline in estimated GFR (r = 0.26, P = 0.003, adjusted), as well as the higher pressure pulse predicted an increased risk to develop end-stage renal disease: adjusted HR of 1.2 (95%CI: 1.1-1.4, P = 0.011)[34]. In addition, a cohort study of 18 T1DM patients followed for 8 years has shown an association between the expansion of the cortical interstitial volume fraction and PA1-activity and VCAM levels[35].

It is worth noting that changes in endothelial function can be identified regardless of the method used. A sustained hyperaemic stimulation induced by the hand skin heating method, as well as FMD vasodilation, were used to evaluate endothelial dysfunction in T1DM patients with and without microangiopathy and also in healthy controls matched for gender, age and body mass index. It was observed that FMD was lower in the diabetes group compared to controls. Furthermore, the presence of clinical complications was significantly associated with lower FMD and creatinine levels were also negatively correlated with the magnitude of FMD. With regard to the hand skin heating method, it was shown that the radial flow shear stress increased vascular diameter in all groups, however, the amplitude of FMD in diabetic patients were significantly lower than in the control group[36]. This dataset demonstrate the importance of endothelial aggression factors as potential markers of vascular injury.

More recently, longitudinal studies have sought to identify markers of endothelial dysfunction as predictors of long-term cardiovascular events. In a prospective study, T1DM patients with persistent normoalbuminuria and nephropathy, without previous cardiovascular events, were followed for a mean period of 12.3 years. The plasma levels of soluble receptor for advanced glycation end products (sRAGE) and other biomarkers were measured at baseline. High levels of sRAGE as a reflection of RAGE expression, was associated with greater incidence of fatal or nonfatal cardiovascular disease, as well as all-cause mortality. Furthermore, there was a significant association between levels of sRAGE and GRF in patients with nephropathy[37]. These authors, in a prospective study with a similar sample, showed that higher plasma levels of the pro-inflammatory cytokine high -mobility group box 1 was an independent predictor of fatal and non-fatal cardiovascular events and also a high-risk marker for all causes of mortality[38].

According to a recently published review, the mechanisms of endothelial dysfunction and ischemic response in diabetes mellitus is complex, involving inflammation, intercellular signaling peptides and proteins, cell angiogenic potential, among others[39]. It is noteworthy that a prospective study demonstrated a decrease of EPCs in children with T1DM, as well as the association between better glycemic control and increased EPCs after an one-year follow-up, suggesting that knowledge of this mechanism may be a way of mediating the high cardiovascular risk in these patients[40]. Therefore, more knowledge on the balance between vascular homeostasis and cardiometabolic risk factors will certainly improve the monitoring of diabetic patients and reduce vascular complications and consequently morbidity.

CONCLUSION

In summary, the pathogenesis of endothelial dysfunction

in T1DM is complex and involves several mechanisms such as inflammation, oxidative stress, interaction between insulin and C peptide, decreased number of endothelial progenitor cells among others. The prognostic value of assessing endothelial function as a marker of cardiovascular morbidity and risk has been demonstrated by cross-sectional and prospective studies with long follow-up, using various methods to identify subclinical atherosclerosis and endothelial dysfunction. The dataset demonstrate that regardless of the method used, impairment of endothelial function is a predictor of risk for cardiovascular disease and nephropathy. This knowledge suggests that new preventive and therapeutic interventions should be recommended early in order to decrease morbidity in this high-risk population.

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P- Reviewer: Cosmi E, Okumura K **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Advances in management of type 1 diabetes mellitus

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Received: May 13, 2014 Revised: June 18, 2014
Accepted: July 17, 2014
Published online: October 15, 2014

Abstract

Treatment of type 1 diabetes mellitus has always posed a challenge to balance hyperglycemia control with hypoglycemia episodes. The quest for newer therapies is continuing and this review attempts to outline the recent developments. The insulin molecule itself has got moulded into different analogues by minor changes in its structure to ensure well controlled delivery, stable half-lives and lesser side effects. Insulin delivery systems have also consistently undergone advances from subcutaneous injections to continuous infusion to trials of inhalational delivery. Continuous glucose monitoring systems are also becoming more accurate and user friendly. Smartphones have also made their entry into therapy of diabetes by integrating blood glucose levels and food intake with calculated adequate insulin required. Artificial pancreas has enabled to a certain extent to close the loop between blood glucose level and insulin delivery with devices armed with meal and exercise announcements, dual hormone delivery and pramlintide infusion. Islet, pancreas-kidney and stem cells transplants are also being attempted though complete success is still a far way off. Incorporating insulin gene and secretary apparatus is another ambitious leap to achieve insulin independence though the search for the ideal vector and target cell is still continuing. Finally to stand up to the statement, prevention is better than cure, immunological methods are being investigated to be used as vaccine to prevent the onset of diabetes mellitus.

Key words: Type 1 diabetes advances; Insulin analogues; Closed loop system; Continuous glucose monitors; Insulin gene therapy

Core tip: As therapy of type 1 diabetes poses important challenges because of life long insulin dependence,multiple injections, excursions in glucose values and inability to simulate the pancreas, newer modalities of therapy are emerging. Hence, this is the right time to review developments in this front. This review conjures up recent advances in continuous glucose monitors, closed loop systems, insulin analogues, insulin gene therapy, transplantation and immunological vaccination.

Original sources: Aathira R, Jain V. Advances in management of type 1 diabetes mellitus. *World J Diabetes* 2014; 5(5): 689-696 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/689.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.689

INTRODUCTION

The year 1923 is a watershed in the history of diabetes mellitus when insulin was discovered by Banting and Best[1]. Today the world has come a long way from that, but living with type 1 diabetes still remains akin to a tight rope walk, balancing between hyperglycemia and hypoglycemic episodes. Multiple injections, strict control on food and exercise are herculean tasks to deal with, especially in children. Hence, the need for better therapies is warranted and they have thus evolved from nascent stages to actual usage.

The incidence of type 1 diabetes varies among different countries, which reflects the roles played by genetic

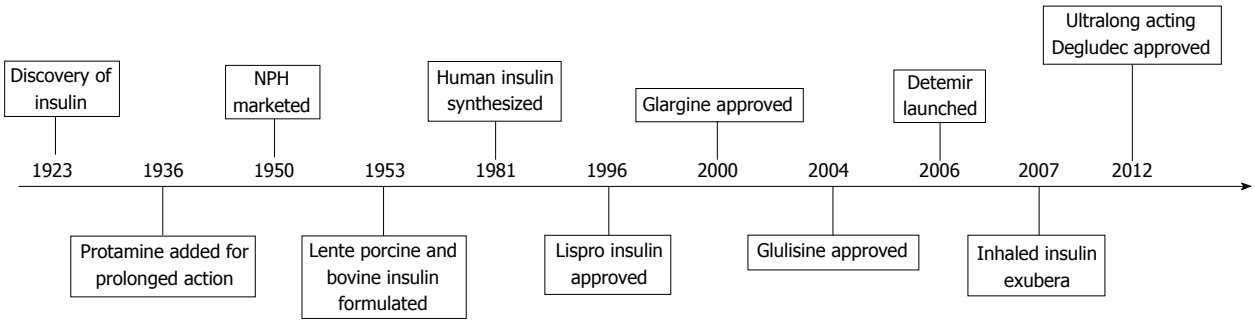

Figure 1 Time line of Insulin and its analogues. NPH: Neutral protamine hagedorn.

and environmental factors in the ultimate expression of the disease. It varies from 57.4 cases/100000 per year in Finland to 0.6 cases/100000 per year in India[2]. The fact that there is a rising trend in the number of children diagnosed to have type 1 diabetes is supported by a number of studies. Whether this can be attributed to an absolute increase in the incidence of the disease is still under speculation because the proportion of children with highest risk human leukocyte antigen haplotypes have decreased and hence, the changing environmental patterns may rather be uncovering the latent genetic factors to cause earlier expression of the disease[3]. The changing epidemiology is bringing more and more children to us to care for. Thus, unveiling newer and better therapies becomes an onus on us.

In this chapter, we shall be presenting a brief overview of the recent advances in the management of type 1 diabetes, including newer insulins, newer insulin delivery options, hypoglycemia prevention through use of technology and lastly, advances in the field of "curing" diabetes through transplant and gene therapy.

ADVANCES IN INSULIN

The quest for the ideal insulin has led to the discovery of a variety of analogues to match the mighty pancreas and yet, many lacunae are left to be filled. The timeline of important events in the history of insulin is presented in Figure 1.

Insulin analogues were designed to overcome the problems of poor stability and erratic absorption profile of the preceding generations of insulin.

Short acting insulin

Insulin lispro: Short acting insulin is necessary to deal with meal time hyperglycemia. Insulin Lispro which was approved in 1996 has rapid onset of action and shorter duration so that post prandial hypoglycaemia can be prevented. The inversion of proline at position 28 with lysine at position 29 allowed insulin to exist more in the monomeric form that is easily absorbed which could counteract meal time hyperglycemia without causing prolonged hypoglycaemia. The modification in the amino acid sequence did not alter the receptor binding and hence, is as effective as regular insulin[4].

Insulin aspart: Substituting proline at position 28 with aspartic acid formed insulin aspart which is also short acting due to absence of hexamer formation. Immunogenicity and teratogenicity profile was similar to regular insulin[5].

Insulin glulisine: This is the newest addition to the list of short acting insulin produced by substituting asparagine at position B3 by lysine and lysine at position B29 by glutamine. It is unique in action by causing phosphorylation of Insulin Receptor Substrate 2. Increased binding to insulin like growth factor (IGF) 1 receptor and mitogenic activity has however, raised concerns over its tumorigenic potential which needs further evaluation[6]. Food and drug administration (FDA) approval has been obtained for use of glulisine in children > 4 years.

Long acting insulin

Isophane, Lente and Ultralente failed to ensure long time control of glucose with minimum variations and hence, they made way for newer long acting insulins.

Insulin glargine: Amino acid alterations brought about a change in pH from 5.4 to 6.7 that made glargine poorly soluble at physiological pH. The stability of its hexameric structure prevents rapid absorption from subcutaneous tissue and its activity is maintained for 11 to 24 h. Glargine also has affinity to the IGF 1 receptor making it mitogenic, but the clinical significance of this finding is still questionable[7]. Safety in the pediatric age group has been established but due to the acidic pH burning sensation has been reported in some children.

Insulin detemir: Detemir binds reversibly to albumin and undergoes a slow release process as only free detemir is biologically active. Onset of action is within 1 to 2 h and lasts for 24 h. Peakless activity ensures stability[8]. Detemir shows more reproducible pharmacokinetics in children than glargine[9]. The United States FDA has approved the use of Detemir and Glargine only in children > 6 years.

Insulin albulin: As the name suggests, insulin albulin has been developed by directly fusing single human insulin gene to human albumin gene that makes this analogue long acting. The peakless effect makes albulin a potential

Figure 2 Dr. Arnold Kadish with the first insulin pump. (Courtesy: www.medscape.com).

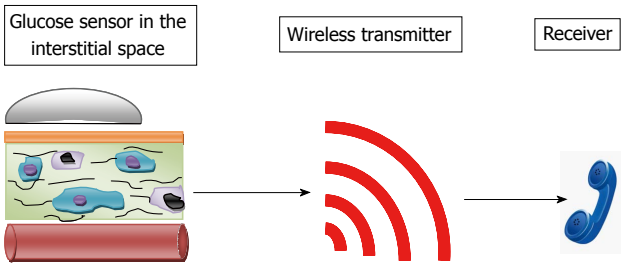

Figure 3 Schematic representation of continuous glucose monitors.

agent for long term glycemic control. The affinity of albulin to IGF 1 receptors is less compared to other analogues which makes albulin less likely to trigger mitogenesis[10]. Insulin albulin still has to evolve to enter clinical application.

Insulin degludec: Approved in 2012, Insulin degludec shows a flat profile upon injection with a half-life of 25 h, enabling once in 3 d injection. The dihexamers associate with each other to form multi hexamers that slowly form monomers and enter the bloodstream. When compared to other long acting insulins, degludec shows much lower variability in day to day glucose levels. Trials investigating degludec have also included children and adolescents. Nocturnal Hypoglycemia, which is the bottle neck in intensive glucose lowering, is reported to be up to 25% lower with degludec[11]. Increase in adverse cardiovascular events is a concern with degludec and use in pediatric age group is not yet approved.

Inhaled insulin

The search for alternative routes of delivery of insulin paved way to the discovery of inhaled insulin Exubera that was approved in 2006, but withdrawn from the market a year later due to poor sales. It was thought that the large surface area of the lungs would facilitate better absorption. However, bioavailability was found to be only 10% and so higher doses were required. Unpredictable absorption patterns that varied with age, respiratory tract infection and smoking form important hurdles for lungs to be the route of choice[12].

Despite the initial enthusiasm with oral insulin which was considered as the "holy grail" for treating diabetes, it remains an enigmatic target due to enzymatic digestion of insulin and inadequate intestinal absorption.

Buccal and skin patches are also candidate routes for delivering insulin that await further research.

INSULIN PUMPS

Parallel to the advancements in insulin, the modes of delivery also underwent considerable changes in the last 50 years. The first pump designed by Dr. Arnold Kadish in 1963 was bulky and had to be worn like a backpack as in Figure 2. It was replaced by the "big blue brick" model which again became obsolete due to inaccuracies. All the early models could only provide a single basal delivery rate and had to be programmed frequently. The technological boom that accompanied the dawn of the 20th century brought about further developments and today we have insulin pumps that are convenient, small, accurate and adjustable.

CONTINUOUS GLUCOSE MONITORS

Fear of hypoglycemia is recognised as the most important road block in the path to achieving good glycemic control. Continuous blood glucose monitoring system is an important aid in the management of type 1 diabetes and an essential prerequisite for closed loop systems. The superiority of Continuous glucose monitors (CGMs) over self-monitoring of glucose in reducing the time spent in hypoglycemia has been proven beyond doubt[13].

The basic structure of a CGM consists of a sensor, wireless transmitter and a receiver as in Figure 3.

Sensor provides real time blood glucose levels and typically consists of a membrane layer, electrode and enzyme matrix. It works on the same principle as the conventional glucose monitors using the glucose oxidase catalysed oxidation of glucose to produce hydrogen peroxide that generates an electric current at the electrode[14]. The membrane layer forms a barrier between the electrode and the surrounding tissues, which mandates adequate permeability to glucose and oxygen. Sensors are inserted subcutaneously and detect glucose concentration in the interstitial compartment. In the earlier versions, blood glucose values were stored and had to be downloaded to view the level of control retrospectively. The present CGMs have sensors that display the glucose values in real time which enables the user to take appropriate steps in case of skewed values. The CGMs are also equipped with systems that would alert the user when values are above or below the set thresholds. The receiver may either be a display device to be worn like a pager or may be connected to an insulin pump.

A drawback that has emerged with CGMs is bioinstability. Sensors become unstable secondary to inflammatory reaction, granuloma formation, blood clots, *etc*[15]. This brings about drifts in glucose values and a need for intermittent calibration with conventional blood glucose

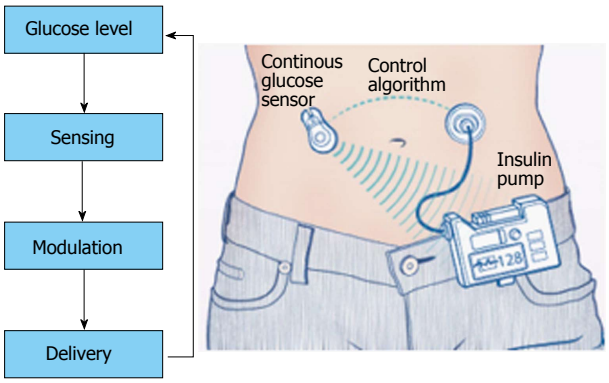

Figure 4 Principle of closed loop system.

measurements. Coating of the membrane layer with silicon oxide nanoparticles containing Polyethylene Glycol has been found to prevent bioinstability of sensors[16]. Further research is ongoing to discover the most appropriate material to coat the sensors. Another innovation that has been successful is replacement of electrochemical sensors with fluorescent sensors. When glucose binds to the receptors, the fluorophore fluoresces brightly. These sensors are highly accurate even with extreme values of glucose[17]. Despite these refinements, there are two important shortcomings with the CGMs. First, the interstitial glucose measurement does not exactly reflect the blood glucose concentration. Second is the time lag due to glucose transport to the interstitium and sensor processing. The CGMs lag behind blood glucose by an average of 4 to 10 min[18].

Another method of blood glucose monitoring that had emerged in 1999 was the Glucowatch Biographer. This device was worn like a wristwatch. It used the process of reverse iontophoresis to stimulate the secretion of subcutaneous fluid, and glucose content was measured using a biosensor unit. There was good correlation with the blood glucose monitoring devices[19]. However, skin irritation and false alarms were obstacles to the widespread clinical use of this device.

A recently developed non-invasive CGM device named HG1c uses the principle of Raman spectroscopy where a painless pulse of monochromatic light is transmitted into the skin, and the scattered light is detected for the determination of glucose levels. This device can be worn on the abdomen like a band and measures blood glucose levels every five minutes. The sensor transmits data to a smartphone which is also enabled with alarms during periods of glucose excursion[20]. A similar iPhone operating system-enabled smartphone-based Wireless Smart Gluco-Monitoring system has also been developed[21].

Many smartphone based glucose monitors and applications are helping to make the life of a diabetic patient easier. These allow the user to enter diabetes related data like carbohydrates and water consumed, insulin dose taken, duration of exercise, *etc*. Based on the information given these apps can also calculate the amount of insulin required. A device named Eyesense is under development which will be able to determine blood glucose level using a small photometer implanted in the interstitial fluid under the conjunctiva[21].

CLOSED LOOP SYSTEMS

The idea of closed loop systems came into vogue as the repeated discrete subcutaneous doses caused fluctuating insulin and in turn glucose levels. Blood glucose concentration stands on a delicate balance between caloric intake and expenditure which is modified by the insulin doses that necessarily do not mimic the original pancreatic secretion. As the CGMs started providing real time feedback of the glucose levels, the extreme variations were uncovered. The concept of artificial pancreas surfaced when CGMs were linked to insulin pumps as Continuous Subcutaneous Insulin Infusion gained acceptance from the 1990s[22]. The principle of closed loop systems is simple as shown in Figure 4.

In contrast to the pre-programmed insulin pumps, closed loop systems modulate insulin delivery at intervals of 1 to 15 min.

The characteristics that are desired in an ideal closed loop system would be the following[23]: (1) Response to glucose levels in a highly specific way; (2) Response within a timescale of minutes; (3) Monitoring within the visceral region; (4) Pulsatile output to avoid desensitization of insulin receptors; and (5) No chemical modification of insulin.

The backbone of the closed loop system is the control algorithm. Control algorithms direct insulin delivery as per glucose levels and account for measurement errors and kinetic delays.

There are two categories of control algorithms: (1) Proportional Integral Derivative (PID); and (2) Model Predictive Control (MPC).

PID

The schema of PID is given in Figure 5. The PID was one of the most initial algorithms developed for artificial pancreas. The proportional component detects deviations from target glucose, integral component measures the area under the curve between the measured and target levels and the derivative component assesses the rate of change of measured glucose levels. However, PID is rather a reactive algorithm which implies that skewed values of glucose cannot be prevented but can only be shortened in duration because the PID responds to observed glucose levels. Adding announced meals to the algorithm or patient directed insulin boluses can overcome hyperglycemia but hypoglycemic episodes may not be prevented.

MPC

This is a proactive algorithm because it can forecast the blood glucose values from the current concentration and is designed in such a way that it brings the forecasted glucose closer to the target glucose values. Based on the current glucose levels further insulin delivery is planned but after the first step is executed the system is reassessed and

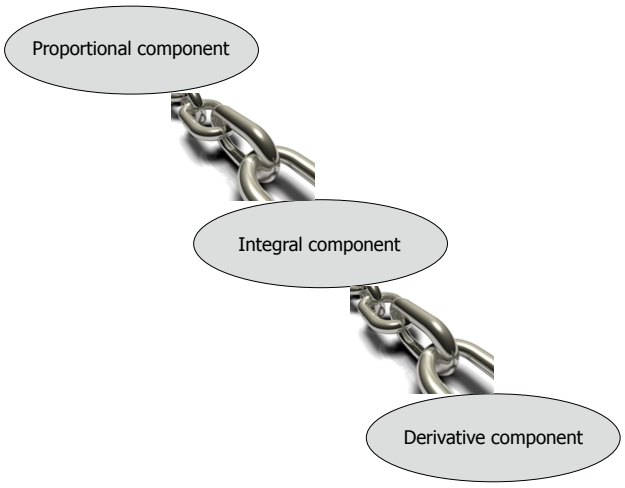

Figure 5 Components of proportional integral derivative.

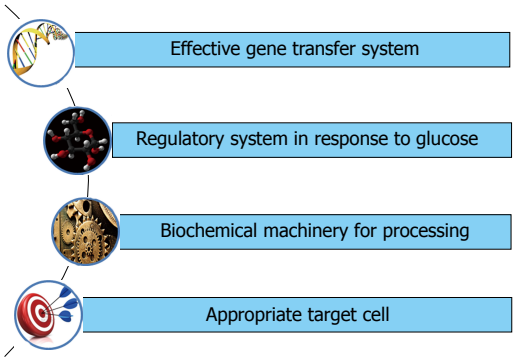

Figure 6 Requirements for insulin gene transfer.

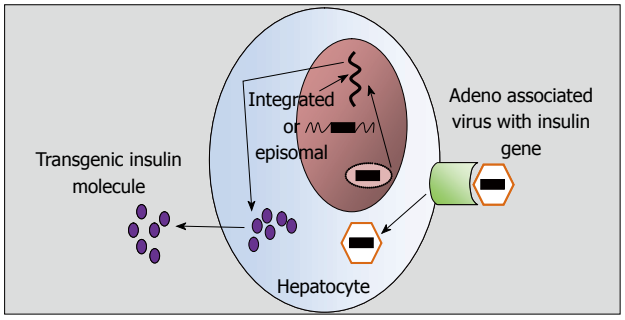

Figure 7 Insulin gene insertion with adeno associated virus.

further delivery is planned. This enables a step by step assessment and reaction, yet in a proactive manner. In this way MPC can prevent hypoglycemic episodes and reduce the time spent in hyperglycemia. MPC can efficiently deal with meals and exercise without any additional inputs[24]. MPC also has capabilities to learn the patient's routine to adjust the insulin delivery based on this information using the run to run control algorithms and also optimize according to circadian fluctuations[25].

Innovations in closed loop system

The inherent disadvantages of interstitial insulin infusion account for the delay in responding to post prandial hyperglycemia. Hence, systems have been developed for adding meal announcements to cause priming.

Intensification of insulin delivery saw hypoglycemia as the major barrier which induced development of dual hormonal pumps employing glucagon along with insulin. Glucagon has been the choice as it is a fast acting counter regulatory hormone to insulin and is found to be deficient in type 1 diabetes patients. Glucagon has enabled to close the glucose-insulin loop in the initial studies[26].

Intraportal or intraperitoneal insulin infusion to mimic the natural secretory pathway is another gate that has been opened for better control of blood sugar. However, the invasive procedure involved in placing the device and risks of infection are the hurdles to its more widespread usage[27].

"Low Glucose Suspend" is another feature to combat hypoglycemia as the pump would automatically stop insulin infusion for up to 2 h when hypoglycemia is detected which is of benefit especially during nocturnal hypoglycemic episodes[28].

Pramlintide is an amylin analogue that delays gastric emptying and reduces glucagon secretion. Pramlintide infusion along with insulin is found to enhance peripheral tissue sensitivity to insulin[29].

INSULIN GENE THERAPY

Gene therapy is the fancy word for most diseases without a cure and so it is for diabetes also. Insulin gene therapy envisages introduction of insulin secretory machinery into non beta cells. The requirements for insulin gene transfer are schematically represented in Figure 6.

Gene transfer system

Gene transfer can be achieved by viral or non viral vectors. Among non viral vectors direct injection of DNA, electroporation and gene gun methods were tried but gene expression was transient. Retro virus, adeno virus and adeno associated virus have been looked upon as the living carriers of the insulin gene (Figure 7). Problems are galore even with these viral vectors. Retro viral vectors integrate at random sites, have limited insertion capacity and infect only proliferating cells. Adenoviral vectors remain as extra- chromosomal DNA and sometimes activate cellular immune response to viral proteins.

Glucose responsive insulin production

Under normal circumstances insulin biosynthesis is regulated at the translational level which is rapid enough to react to physiological changes. Transcriptional control supplements the translational regulation. To ensure glucose responsiveness, glucose responsive promoters are linked to the insulin producing gene. However, introducing promoters alone may not be sufficient as translational regulation is difficult to be mimicked in a non-beta cell[30]; and since insulin release is controlled at the transcriptional level the rapidity of the response would

be compromised.

Biochemical machinery for processing

Proinsulin is converted to insulin by endoproteases PC1, PC2 and an exopeptidase, carboxypeptidase H which is another example of translational control[31]. In non beta cells the generic proprotein convertase Furin can cleave pro-insulin if appropriate cleavage sites are introduced by mutation but mutated pro- insulin may induce immune attack[32].

Appropriate target cell

An ideal target cell ought to have all beta cell characteristics but has to be free from immune attack. This statement seems utopian as the sophisticated machinery in the beta cell for insulin synthesis and release according to the metabolic needs is not to be easily found in any other cell type. Hepatocyte stood out as a good option as it is enabled with glucose sensing system and glucose regulated promoter. Unfortunately there are no processing enzymes and exocytosis system[33]. The pituitary cell on the other hand, has processing enzymes and exocytosis system but lacks glucose sensing system. Myocytes are also among candidate target cells. K cells, endocrine cells in the gut that secrete incretins, are endowed with glucose sensing system, glucose regulated promoter, exocytosis system and processing enzymes. Genetically engineered K cells have been shown to produce enough insulin in a glucose regulated manner in murine models though tumor cell lines were used. Though the ideal target non beta cell still remains elusive, the K cells form a promising option[34,35].

TRANSPLANTATION

Whole pancreas transplant

Despite developments in closed loop systems and encouraging results from insulin gene therapy, completely mimicking the beta cells still remained a distant dream. Thus, pancreas transplant was considered as a viable option. Whole pancreas transplant was tried initially in patients requiring kidney transplant but complications were galore like pseudocyst, fistula, thrombosis and pancreatitis. Moreover, transplanting the whole pancreas when the patients were only in need of the islets of Langerhans which constitute a meagre 2% of the pancreatic mass was like losing the battle for want of a horse shoe nail[36].

Islet cell transplant

In addition to transplanting only the endocrine component, islet cell transplantation is minimally invasive and is associated with lower morbidity. After pancreas retrieval, the islets are isolated and cultured which is the most formidable step in the whole procedure. The most commonly used anatomical site for islet transplant is the liver due to the convenience of access and good entrapment and engraftment in the sinusoids though spleen, renal capsule and the gonads have been tried[37]. Islet cell transplantation done in animals resulted in universal reversal of diabetes but reproduction of these results in human beings was a Himalayan task in the 1990s as only 11% achieved insulin independence. However, in 2009, the Collaborative Islet Transplant Registry reported that the overall incidence of sustained graft function was 77% after first 6 mo, 66% after 1 and 45% at 3 years[38]. Though independence from exogenous insulin can be achieved, extrapolation of results from studies done in adults to children with type 1 diabetes mellitus (T1DM) would be a precocious decision and awaits more research.

Stem cell therapy

The interest stem cell therapy created in almost all chronic diseases is also reverberating in type 1 diabetes. Generation of sufficient mass of beta cells, releasing insulin in response to physiological signals and protection from autoimmunity are the most important challenges. Stem cells can be converted to beta cells by sequential transient activation of specific transcription factors like Pa x 4, Nk x 6.1 and Nk x 2.2[39]. The possibility of teratogenicity with embryonal stem cells makes mesenchyme derived stem cells a better option. An alternative approach is by neogenesis of beta cells from mature beta cells with the use of GLP analogue (Exendin), Epidermal Growth Factor and gastrin. The common endodermal origin of pancreas, liver and small intestine allows trans-differentiation of any of these cell types to beta cells[40]. Trans-differentiation involves reprogramming mature cells by certain transcription factors into alternate developmental lineages.

IMMUNOLOGIC VACCINATION

The principle behind this model is to induce lymphocytes against a specific antigen in such a way that on encountering that particular epitope the lymphocytes would induce cytokines that suppress autoimmunity like interleukin 4 that are produced by Th1 cells. Insulin given orally and subcutaneously in mice models prevented T1DM[41]. Replicating these findings in humans will take time but these provide some light at the end of the tunnel.

CONCLUSION

Novel therapies are continuing to emerge for the ultimate cure of type 1 diabetes, but emulating the intricate control system of the beta cell that is tailor made for minute to minute control of blood sugar is a difficult goal to attain. We hope that sustained efforts toward this distant goal will provide the elixir for millions of children with T1DM.

Continuous glucose monitors have evolved from retrospective display to real time monitors enabled with alarms connected to smartphones and to more non-invasive methods. Closed loop systems have been undergoing developments to simulate the pancreas by incorporating better sensors, feedback, control algorithms and response. Newer insulin analogues have more predictable half-life

and activity. Inhalational, buccal and transdermal delivery routes are awaited for clinical application. Insulin independence is aimed at by incorporating insulin gene into non beta cells with reliable glucose response apparatus. Islet cell transplantation is also continually transforming to reach the point of complete cure. Immunological vaccination is in its nascent stages to prevent the occurrence of type 1 diabetes.

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P- Reviewer: Georgescu A, Romani A, Vorobjova T
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Intensive diabetes management and goal setting are key aspects of improving metabolic control in children and young people with type 1 diabetes mellitus

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Received: August 5, 2014 Revised: October 5, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

Diabetes control in children remains poor in spite of advances in treatment for last 10 years. The aim of this review was to look at various aspects of intensive therapy in the management of type 1 diabetes such as insulin regimes, role of target setting, psycho-educational approaches and self-management. To achieve good metabolic control, clear goal setting with adequate support for self-management are essential. Psycho-educational and behavioural interventions aimed at specific areas of management have shown significant improvement in quality of life and diabetes control.

Key words: Type 1 diabetes; Children; Metabolic control; Intensive; Management; Goal setting

Core tip: The aim of diabetes treatment is to maintain normoglycaemia in order to prevent long term complications. Insulin is the mainstay of diabetes treatment and is delivered by various regimens. Superiority of one regimen over the other is not established. Newer techniques with sensor augmented pumps have shown improvement in the diabetes control. Other aspects of intensive treatment are goal setting and adequate multidisciplinary support for self-management. Self-management is necessary to achieve the goals of diabetes treatment. Interventions based on clear psycho-educational principles are shown to be effective in improving outcomes.

Original sources: Soni A, Ng SM. Intensive diabetes management and goal setting are key aspects of improving metabolic control in children and young people with type 1 diabetes mellitus. *World J Diabetes* 2014; 5(6): 877-881 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/877.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.877

INTRODUCTION

Type 1 diabetes is characterised by autoimmune destruction of the β cells leading to insulin deficiency. It accounts for 90% of childhood diabetes in the western world. The incidence has been increasing over past 2 decades and poses a global challenge[1]. The aim of diabetes management in children is to achieve near normoglycaemia without major hypoglycaemic episodes and to prevent long term complications associated with hyperglycaemia[2].

Early normalisation of blood sugars with intensive insulin therapy might lead to improved long term control and higher endogenous insulin production 1 year after the diagnosis[3]. Good glycaemic control in patients with Insulin Dependent Diabetes mellitus delays the onset and slows the progression of long term complications. Several approaches are taken when aiming for low glucose targets. The Diabetes Control and Complication trial

Table 1 Review of studies comparing different insulin regimens

Ref.	Method/population	Outcome
de Beaufort *et al*[10]	Observational cross-sectional international study/2036 patients(11-18 yr)	No improvement in glycaemic control over a decade Those on twice daily free mix had significantly better control and the ones on twice daily injections had the worst HbA1c
Holl *et al*[11]	Multicentre Observational study/872 patients (11-18 yr)	Deterioration in metabolic control in all three groups over 3 yr period One group had moved from twice daily to multiple injections
Haller *et al*[12]	Observational Study (enrolled patients were on preferred regimes from 12 paediatric endocrinologists)/229 patients (9-15 yr)	Increased number of insulin types correlated with increased HbA1c
Nordly *et al*[13]	Multicentre cross sectional sudy/874 (< 16 yr)	Children with 2 injections a day had significantly better control than children on 3 or four injections a day
Paris *et al*[14]	Multicentre cross-sectional study/2743 patients (< 20 yr)	Insulin pump users had better control. No difference between MDI or 2-3 injections a day
Jakisch *et al*[15]	Multicentre matched pair cohort analysis, comparing CSII to MDI/434 matched pairs	Significantly better HbA1c in CSII group after 1 yr but subsequently no difference at 3 yr

MDI: Multiple daily insulin; CSII: Continuous subcutaneous insulin infusion; HbA1c: Hemoglobin A1c.

(DCCT) clearly showed that intensive therapy aiming for lower target blood sugars measured by lower mean glycosylated haemoglobin A1c (HbA1c) reduced the risk for onset and progression of diabetes complications[4]. However, intensive treatment does not just include intensive insulin regimes but patient education, counselling and effective diabetes self-management[5]. It can best be provided with well-sourced multidisciplinary team with focus on treatment goals and regimes, self-management, patient education and frequent clinic visits[6]. There is considerable diversity in delivery of these interventions and it has been a challenge to find practical, clinic based interventions that can provide improvement in HbA1c similar to those achieved in DCCT. Hvidoere study group have demonstrated that the clinical and metabolic goals or targets are more important in determining the outcomes than the therapeutic regimen on its own. Self management, structured education for the patient and family, and close telephone contact with the diabetes team are also associated with reduced hospitalisations and emergency room visits[7].

The purpose of this review is to examine the key aspects of improving metabolic control in children and young people with diabetes who have characteristics and needs that dictate different standards of care. We will look specifically at the impact insulin delivery and regime, self-management of diabetes which includes psychological intervention, self-education programmes and goal setting in improving outcomes.

INSULIN DELIVERY AND REGIME

Treatment with insulin is the mainstay of therapy in type 1 diabetes mellitus. Many formulations are available but with the advent of newer analogues, they are mainly used in treatment in children. There is no data on the long term benefits of these analogues but they provide more flexibility and some improvement in the care of diabetes[8,9].

The choice of insulin regime depends on the individuals The basal bolus therapy or multiple daily insulin (MDI) regimes consists of long or intermediate acting insulin is given once or twice a day with boluses of rapid acting insulin analogue with meals. Insulin pump or continuous subcutaneous insulin infusion (CSII) works on similar principles but delivers short acting analogue continuously with boluses at meal times. After DCCT trial, these modalities have become the norm of diabetes treatment. Other methods include use of pre-mixed insulin which contain fixed ratio mixtures of short and intermediate acting insulins. They are given as two injections a day. Currently, there is no clear evidence that one insulin regime is superior to other on its own[10].

There are various cross-sectional studies looking at different insulin regimes (Table 1) but none of them have found any clear evidence that one is superior over the others.

Insulin pumps

There are several systemic reviews and meta-analysis including a Cochrane review comparing CSII to MDI[16]. Most of them have favoured CSII for better control but recent meta-analysis comparing CSII to MDI showed no significant change in HbA1c from baseline level after 16 wk or more of follow up in children. Overall CSII has been found to yield better quality of life compared to MDI, however benefit to glycaemic control is variable[16,17].

Sensor augmented pump therapy (SAP) which integrated CSII with a continuous glucose sensor. In a comparative meta-analysis sensor-augmented insulin pump use resulted in a statistically and clinically significant greater reduction in HbA1C levels than with MDI or self-monitoring of blood glucose (SMBG) in persons with type 1 diabetes mellitus[17]. Sensor-Augmented Pump Therapy for A1C reduction. STAR 3 study has shown that compared to MDI, SAP offers rapid glycemic advantage in children and adolescents which lasted for the entire year of study phase[18,19].

SMBG is the key to achieving main goals of insulin therapy. Several studies have established that frequency

of SMBG is directly proportional to improved HbA1c levels[12,20].

More recently continuous glucose monitoring (CGM) has been used and can provide information on trends of blood glucose levels. It is considered to be useful for children with poorly controlled diabetes. Recent Cochrane review has shown that there is limited evidence of improved glycaemic control in patients with poorly controlled diabetes. But the review found larger decline which was statistically significant in HbA1c for real-time CGM users starting on insulin pump therapy(sensor augmented pumps) compared to patients using MDI and SMBG (conventional therapy)[21].

GOAL SETTING AND PSYCHOLOGICAL INTERVENTIONS TOWARDS SELF MANAGEMENT

Specific goal setting is an encouraging way of improving adherence to diabetes management in young people[22]. As parental support and involvement is associated with better management of diabetes in children and adolescents, their perception of goals for optimal management of diabetes is associated with actual control achieved in children[23]. Hvidoere study group has documented persistent inter-centre differences in the mean HbA1c over 10-year period in spite of changes to the insulin regimes[10]. They concluded that target setting might me the most influential factor in lowering the HbA1c[24]. Key findings from their work suggests that best metabolic results are obtained by physicians who target driven and teams and families have unanimity of purpose[7].

It is important to have necessary self-management skills in order to achieve goals of diabetes therapy. Diabetes self-management is the process of providing the person with diabetes education, knowledge and skills needed to successfully manage diabetes[25]. It is multi-dimensional and refers to the young persons or/and parents sharing responsibility and decision making for achieving optimal control[26]. Goals for self management varies considerably by age, development, family characteristics, duration of diabetes and lifestyle[27,28]. Adolescence could be a challenging time in control of diabetes. It has been recognised that diabetes control tend to decline during this period[29]. As young people strive for autonomy, social influence and peer pressure with desire to fit in can be higher priority than diabetes management for some young people[30,31]. Various psychological and educational interventions are used to empower the young person with necessary self-management skills but efficacy of one over another is not established. Wysocki *et al*[32] found that youths with suboptimal pre-treatment status with high autonomy to maturity (AMR) did better with intensive treatment over 18 mo period compared to the ones who had low AMR and better HbA1c. An integrated review in 2011 demonstrated that there is a clear relationship between self-management and metabolic control but there is multitude of factors playing part[28].

Research has also shown that there is an association between psychosocial factors and metabolic control in a large international cohort of adolescents with type 1 diabetes mellitus[33] Good metabolic control is associated with better quality of life in adolescents[34,35]. It is also associated with families of children with better control reporting lower disease burden. Behavioural interventions for young people with diabetes and their parents have demonstrated improvement in adherence of treatment[36]. Interventions based on clear psycho-educational principles are most effective[37]. In a systematic review of psychological interventions for improving diabetes control, psychological therapies led to significant improvement in glycaemic control in children and adolescent compared to adults[38]. A case study of 9 adolescents with consistently poor control previously has shown has shown marked improvement with coaching[39]. These findings show that assessment of psychosocial factors should be an integral part of the paediatric diabetes care in this population[33,40].

There are various structural education programmes for adults with type 1diabetes which have shown improvement in their control as well as quality of life[41,42]. However, there is need for practical, clinic based educational interventions for children and adolescents. Various trials have reported disappointing outcomes in improving control when applied to families and children in a real life setting[43,44]. The Kids in control of food is a structured education course based on Dose Adjustment for Normal Eating course which is a current adult education programme. The pilot showed significant improvement in quality of life and self-efficacy at 3 and 6 mo. There was no change in glycaemic control overall but improvement trend in those with poorest control[45]. Results of the randomised trial will hopefully give us more information on the effect of highly structured group education on a population with wide range of glycemic control[46].

In a systematic review by Hampson *et al*[37], it was concluded that educational and psychological interventions are most likely to be effective if demonstrate an interrelatedness of various aspects of diabetes management. There is a gap in evidence as no complete understanding of where these interventions to be targeted.

CONCLUSION

Good metabolic control is needed to prevent long term complications of diabetes. It is challenging in the paediatric population to achieve optimal control due to various developmental and psychological factors[47]. Psycho-educational and behavioural interventions play an important role in the diabetes management. However, there is need for practical, cost effective interventions which could be applied to the diabetes population in a clinic setting such as goal setting and psychosocial interventions. Svensson *et al*[48] have reported significant improvement in diabetes control independent of number of injections per day or insulin regimens but thought to be due to increased focus

on treatment goals, glucose monitoring and optimising care in their population over 10 years period. Overall, this review concludes that clear goal setting with good multidisciplinary team effort working together with families and children towards specific targets may be the key to good diabetes control.

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P- Reviewer: Chen SS, Dumitrascu DL, Das UN
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Diagnosis of hepatic glycogenosis in poorly controlled type 1 diabetes mellitus

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Received: August 12, 2014 Revised: October 2, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

Hepatic glycogenosis (HG) in type 1 diabetes is a underrecognized complication. Mauriac firstly described the syndrome characterized by hepatomegaly with altered liver enzymes, growth impairment, delay puberty and Cushingoid features, during childhood. HG in adulthood is characterized by the liver disorder (with circulating aminotransferase increase) in the presence of poor glycemic control (elevation of glycated hemoglobin, HbA1c levels). The advances in the comprehension of the metabolic pathways driving to the hepatic glycogen deposition point out the role of glucose transporters and insulin mediated activations of glucokinase and glycogen synthase, with inhibition of glucose-6-phosphatase. The differential diagnosis of HG consists in the exclusion of causes of liver damage (infectious, metabolic, obstructive and autoimmune disease). The imaging study (ultrasonography and/or radiological examinations) gives information about the liver alterations (hepatomegaly), but the diagnosis needs to be confirmed by the liver biopsy. The main treatment of HG is the amelioration of glycemic control that is usually accompanied by the reversal of the liver disorder. In selected cases, more aggressive treatment options (transplantation) have been successfully reported.

Key words: Hepatic glycogenosis; Type 1 diabetes mellitus; Hepatomegaly; Glycogen; Glucose transporters; Insulin; Glucokinase; Glycogen synthase; Glucose-6-phosphatase

Core tip: This review contain an extensive revision of the case reports described in literature; in particular glycemic control (elevation of glycated hemoglobin, HbA1c levels, presence of ketoacidosis and insulin dosage), imaging studies and bioptic findings are summarized and discussed. The pathophysiological mechanisms behind the accumulation of glycogen in hepatocytes in patient with poorly controlled type 1 diabetes mellitus are described in detail.

Original sources: Giordano S, Martocchia A, Toussan L, Stefanelli M, Pastore F, Devito A, Risicato MG, Ruco L, Falaschi P. Diagnosis of hepatic glycogenosis in poorly controlled type 1 diabetes mellitus. *World J Diabetes* 2014; 5(6): 882-888 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/882.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.882

INTRODUCTION

Primary glycogenosis or glycogen storage disease is a well known hereditary disease affecting liver and muscles, characterized by the presence of hepatomegaly, hypoglycemia, muscle weakness and growth delay. On the contrary, secondary glycogenosis [hepatic glycogenosis (HG)] is less described in the literature, but it may be frequently

observed and underrecognized in type 1 diabetes (T1D)[1]. Mauriac[2] firstly described the syndrome in 1930. The main features in prepuberal children are hepatomegaly with increased liver enzymes, growth impairment, delay puberty and Cushingoid features in poorly controlled T1D[3]. In young adults with T1D the syndrome is uncomplete, and, in fact, only hepatomegaly with increased liver enzymes are present. The latter alterations are often underrecognized or confused with fatty liver disease or non-alcoholic steatohepatitis (NASH), that is common in T2D[4]. In rare cases, glycogen storage hepatomegaly has been described also in T2D[5].

PATHOPHYSIOLOGY

As pointed out by Wasserman[6], 4 grams of glucose circulates in the blood (a small fraction of the body mass) and 100 grams of glycogen are present in the liver. In glucose homeostasis, the liver plays a significant role for synthesis, storage and redistribution of carbohydrates, with opposite effects during hyperglycemic (glucose uptake and glycogen synthesis) and hypoglycemic conditions (glycogenolysis and gluconeogenesis)[7].

The glucose transport into cells is mediated by fourteen members of membrane glucose transporter (GLUT) molecules, divided into three families (Classes 1 to 3). The expression of the GLUTs varies between different cellular subtypes in liver (hepatocytes, endothelial cells, Kupffer cells and cholangiocytes)[8].

The liver is not considered as an insulin-sensitive tissues, such as skeletal and cardiac muscle, brown and white adipose tissue and endothelial cells. In fact, the transport of glucose into the hepatocytes is mainly mediated by the GLUT2 (insulin-independent, low-affinity, high-capacity with a Km of 10-20 mmol/L), but hepatocytes also express lower levels of GLUT1, GLUT3, GLUT4 (insulin-dependent), GLUT8, GLUT9, GLUT10[9-16] (Figure 1).

After the entrance, glucose is available for the intracellular metabolism. Glucokinase is a phosphorylating enzyme, acting with not stringent substrate specificity for glucose (it is able to phosphorylate hexoses like mannose or fructose in addition to glucose), to produce glucose-6-phosphate (G6P)[17]. There are four mammalian isoenzymes (hexokinases I -Ⅳ or A-D), displaying extensive sequence identities[18]. Glucokinase (GCK, or hexokinase Ⅳ or D) has a low affinity for glucose ($S_{0.5}$ approximately equal to 6 mmol/L) and a rate of reaction with sigmoid dependence on intracellular glucose concentration (cooperativity), operating as an ultrasensitive physiological glucose sensor in hepatocytes with non-limiting glucose transport. If blood glucose is below 5 mmol/L (90 mg/dL) there is no significant effect of GCK on G6P production and subsequent steps, ensuring that hepatic glycogen synthesis is only engaged when blood glucose levels are high.

In the human liver, expression of GCK is strictly dependent on the presence of insulin, and the sterol regulatory element binding protein (SREBP1c), a master regulator of lipogenic enzymes, has been proposed to be a mediator of insulin induction of GCK[19].

Moreover, the GCK activity is modulated by the GCK regulatory protein (GCKRP) that binds and inhibits GCK, competitively with respect to glucose[20]. GCK is localized to the nucleus of the hepatocyte, where it is retained by GCKRP, but moves into the cytosol when glucose levels increase.

The hydrolysis of G6P to glucose (the inverse reaction of GCK) is mediated by the enzyme glucose-6-phosphatase (G6Pase), and its deficiency causes the impaired glycogenolysis of one type of the genetic accumulation of glycogen in hepatocytes, previously described by Von Gierke [glycogen storage disease type I (GSD1a)][21,22]. GSD1a has typical hypoglycemic events after a four to six hour fast (differentiating GDS1a from T1D), lactic acidosis, hypertriglyceridemia, and hyperuricemia[23].

The G6P is successively converted into G1P by phosphoglucomutase. Then, uridine diphosphate (UDP)-glucose pyrophosphorylase transforms G1P into UDP-glucose in the presence of uridine triphosphate, releasing inorganic pyrophosphate.

The G6P, after the phosphorylation by GCK, functions as an allosteric activator of the phosphorylated glycogen synthase (GS) for the glycogen synthesis[24]. Insulin significantly stimulates the glycogen synthesis in hepatocytes. Insulin binds the α-subunit of insulin receptor (IR) on the cellular surface of hepatocytes, inducing the dimerization of the α2β2 complex and the tyrosine kinase activity of the β-subunits. Then, the IR is autophosphorylated and the IR activation recruits and phosphorylates several substrates, including insulin receptor substrate 1-4. The downstream signaling proteins activates phosphotidylinositide-3-kinase (PI3K) to protein kinase B (PKB, also known as Akt signaling cascade), a pathway controlled *via* a multistep process[25]. In particular, the activation of PI3K converts phosphatidylinositol (3,4)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The 3-phosphoinositide-dependent protein kinase 1 and 2 (PDK1 and PKD2) phosphorylate and activate PKB/Akt, allowing to bindPIP_3 at the plasma membrane. The activation of PKB/Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3). GSK3 is a negative regulator of GS, through the phosphorylation at COOH-terminal residues. The result of insulin signal transduction is the GS dephosphorylation that activates the enzyme and the glycogen production. The GS is the rate-limiting enzyme for glycogen synthesis and it catalyzes the addition of α-1,4-linked glucose units from UDP-glucose to a nascent glycogen chain[26]. The UDP-glucose is the glycosyl donor in the reaction catalyzed by GS. There are two GS isoforms: the muscle GS (encoded by *GYS1* gene), and the liver isoform (encoded by *GYS2* gene)[27].

Glycogen is a branched polymer of glucose residues connected by α-1,4-glycosidic linkages formed by the enzyme GS and branchpoints formed *via* α-1,6-glycosidic linkages, introduced by the branching enzyme, occurring

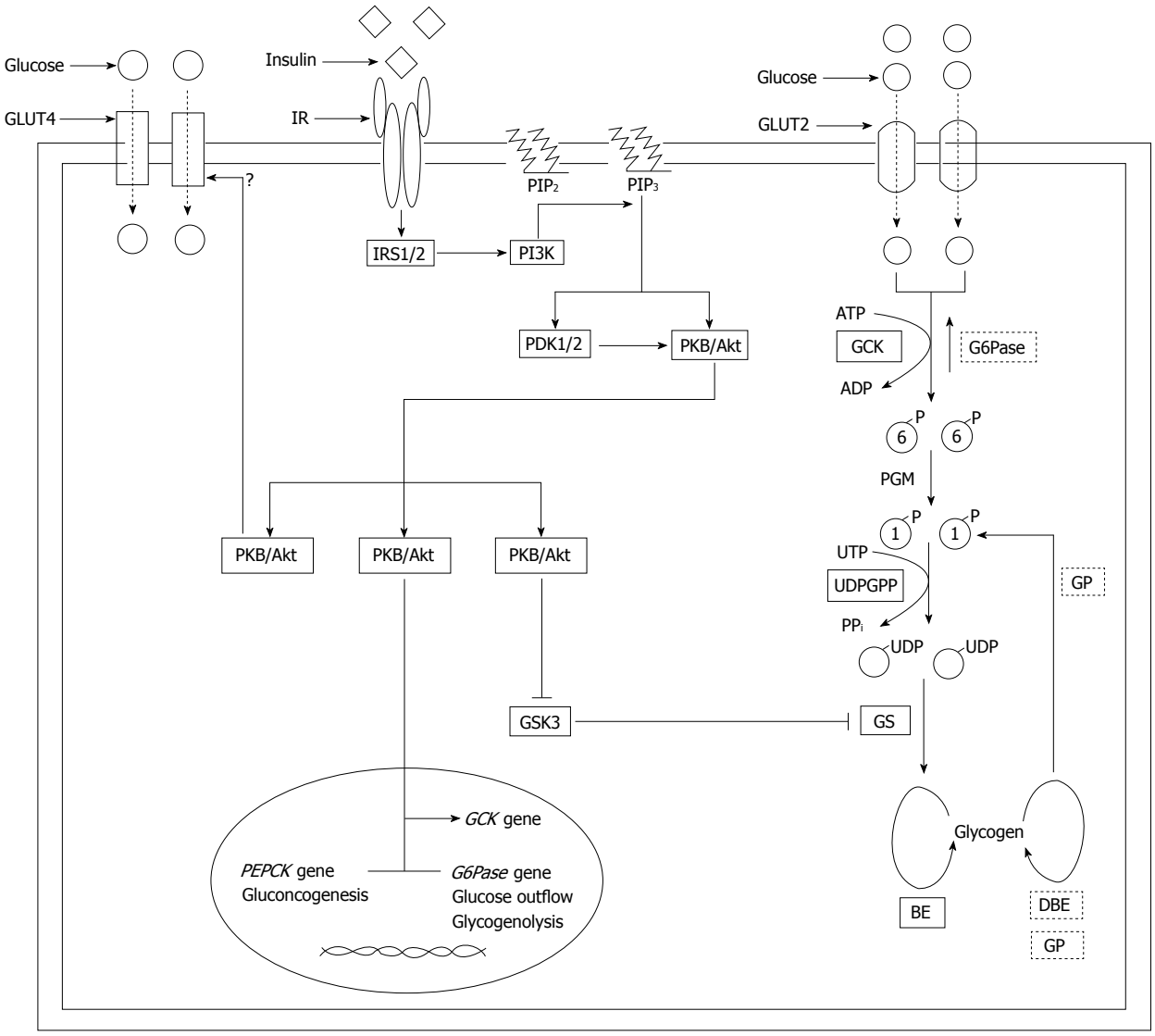

Figure 1 The metabolic pathways of glycogen synthesis in hepatocytes. GLUT: Glucose transporter; IR: Insulin receptor; PIP_2: Phosphatidylinositol (3,4)-bisphosphate; PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate; IRS: Insulin receptor substrate; PI3K: Phosphotidylinositide-3-kinase; PDK1/2: 3-phosphoinositide-dependent protein kinase 1 and 2; PKB/Akt: Protein kinase B; GCK: Glucokinase; G6Pase: Glucosio-6-phosphatase; PGM: Phosphoglucomutase; UDPGPP: UDP-glucosepyrophosphorylase; GP: Glycogen phosphorylase; GSK3: Glycogen synthase kinase 3; GS: Glycogen synthase; PEPCK: Phosphoenolpyruvate carboxykinase; BE: Branching enzyme; DBE: Debranching enzyme; UTP: Uridine triphosphate; PPi: Pyrophosphate.

every 8-12 glucose units.

New glycogen synthesis begin near the plasma membrane, at the periphery of the hepatocyte. Then, glycogen deposits grow from the periphery towards the interior of the cell. Through this way of glycogen deposition, hepatocytes may store large amounts of glycogen.

Glycogen degradation takes place in the reverse order. Glycogen phosphorylase (GP) is the key enzyme in glycogenolysis, yielding G1P[28]. When hepatocytes are depleted of glucose, the GP-mediated phosphorolysis of glycogen proceed from the interior to the exterior of the hepatocyte[29]. Phosphorylase kinase stimulates GP and protein phosphatase 1 inhibits phosphorylase kinase and GP.

Besides stimulating the glycogen synthesis, insulin severely inhibits hepatic glucose output, suppressing gluconeogenesis and glycogenolysis, by inhibiting expression and activity of the key enzymes phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase[30].

The inhibition of gluconeogenesis and glycogenolysis are IR-mediated PI3K and Akt dependent effects. Akt translocates into the nucleus, where it phosphorylates FOXO1 (a member of the O-class of forkhead/winged helix transcription factors), inhibiting *PEPCK* and *G6Pase* gene transcription[31]. Moreover, Akt phosphorylates and inhibits CRTC2, cAMP response element binding protein-regulated transcription coactivator-2, also reducing hepatic gluoconeogenesis[32].

Adolescent diabetic patients with their metabolic activity, dietary intake, and disease state (high frequency of ketoacidosis and increase in exogenous insulin) represents a high-risk subjects, with diabetes control often deteriorating[33].

In T1D patients with poor glycemic control, two

Table 1 Summary of hepatic glycogenosis in type 1 diabetes patients

Ref.	Sex	Age (yr)	BMI	AST (U/L)	ALT (U/L)	HbA1c (%)	Insulin (U/kg)	Glucose (mg/dL)	US exam	CT scan	Biopsy
[51]	M	16	20	66	58	11.1	0.98	198	X		X
[52]	F	17		138	164	12			X		X
[54]	M	19		262	519	12.7[a]				X	X
[55]	F	19	27	98	49	7.9			X	X	X
	M	37		769	844	16			X	X	X
[56]	F	19	23		800	12.2[a]			X		X
[57]	F	3		300	350	9.5[a]	1.5	522	X		No
	M	16		100	200		1.3	810	X		No
[33]	M	14		290	127	13.4	1.6		X	X	X
	F	17		102	147	13.3[a]	1.8				X
	F	16		567	316	12.2[a]			X		No
[1]	F	17	21.4	1620	629	13	0.9		X		X
[58]	M	16	21.1	578	526	11.0[a]					X
[59]	F	22	18.6	1028	365	13.8			X		X
	F	26	23.6	914	307	12.9			X		X
	F	20	21	1310	346	13.6			X		X
[53]	F	29		4000	1900	15.3[a]			X		X
[60]	M	13			1000	13	1.2		X	X	X
[36]	F	20		249	383	13.3[a]				X	X
[61]	F	13			113	8.8[a]		890	X		X
[35]	F	19		83	97	[a]		520			X
	M	12		47	49	13.5[a]		635			X
	F	22		77	48			183			X
	M	8		H	H						X
	F	15		N	N						X
	M	22		360	1100	16.0[a]		404			X
	M	25		1128	1629	10.8					X
	M	16		H	H	[a]					X
	M	20		120	N	9.9		288			X
	F	18		57	N	10.8		137			X
	M	28		1544	1099			H			X
	M	34				10		259			X
	M	16		1354	1413			365			X
	F	23		224	255						X
[41]	F	19			199	14.6[a]				[b]	X

[a]Recent ketoacidosis; [b]Magnetic resonance imaging. H: High level; N: Normal levels; M: Male; F: Female; BMI: Body mass index; AST: Aspartate-aminotransferase; ALT: Alanine-aminotransferase; HbA1c: Glycated hemoglobin; US: Ultrasound; CT: Computed tomography.

combined events are usually present, promoting hepatic glycogen deposition: hyperglycemia (as pointed out by increased blood glucose level and glycated hemoglobin, HbA1c) and consequent large amount of insulin (as demonstrated by elevated insulin dose as UI/kg of body weight/day). In hyperglycemia, glucose passively enters the hepatocytes by insulin-independent GLUT2, and it is rapidly phosphorylated, with inhibition of its release from hepatocytes[34]. The GCK convert the glucose into the G6P, with subsequent trapping in the hepatocyte. Then, an increased insulin administration promotes the polymerization of G6P in glycogen by GS, driving the large amount of glycogen synthesis in the presence of high cytoplasmic glucose concentrations[29]. Therefore, glycogen is trapped within the hepatocytes as a result of a combination of both hyperglycemia and insulin treatment. The consequent liver damage become evident with the blood release of aminotransferases.

Repeated ketoacidosis episodes in T1D increase the risk for hepatic glycogen overload, since diabetic ketoacidosis (a fatal complication of poor controlled diabetes) is usually treated with sustained levels of intravenous insulin (in the presence of high glucose blood concentrations).

DIAGNOSIS

Nowadays Mauriac syndrome during childhood is uncommon especially with the advent of new insulin analogues and intensive insulin regimens. More frequently, patients affected are teenager or young adults and the diagnosis may be difficult[3]. During adulthood, the key symptoms are hepatomegaly, abdominal pain, and other symptoms such as nausea and vomiting. Laboratory findings are high levels of glucose, glycated hemoglobin (HbA1c, demonstrating a poor long-term glycemic control) and aminotransferases [aspartate and alanine, Aspartate-aminotransferase (AST) and Alanine-aminotransferase (ALT), respectively, suggesting liver damage][35]. The range of AST/ALT values is from 47/48 UI/L to 4000/1900 UI/L (Table 1). The investigations about hepatomegaly and elevated aminotransferases include investigations for infectious diseases, metabolic (such as Wilson disease), obstructive or oncologic causes and autoimmune liver tests to exclude all these possible

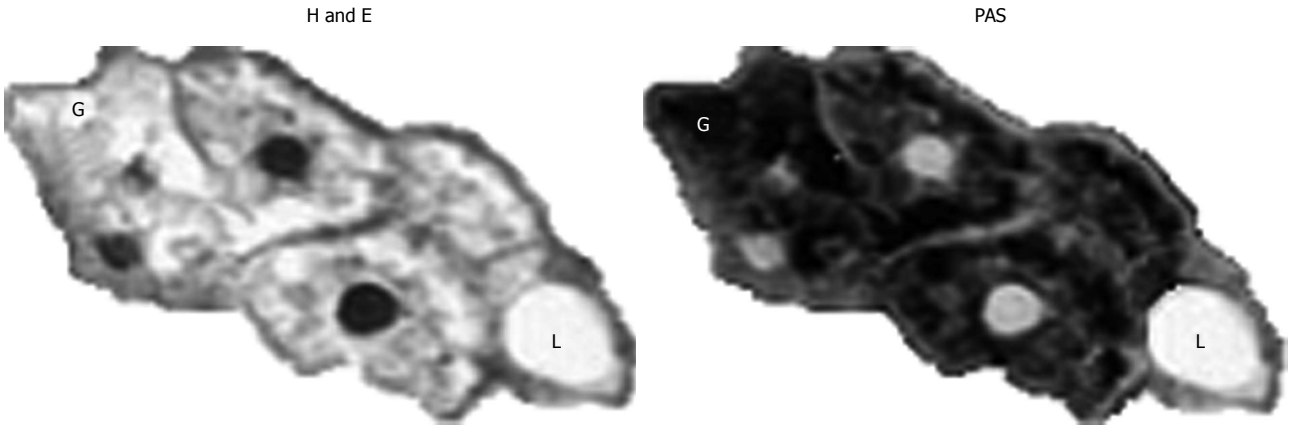

Figure 2 Schematic reproduction of staining with Hematoxilyn and Eosin *vs* Periodic Acid Schiff. The glycogen (G) disappears in H and E whereas it stains (red) in PAS. The presence of lipids (L) in focal vescicular steatosis is demonstrated by lack of staining both in HE and PAS. H and E: Hematoxilyn and Eosin; PAS: Periodic Acid Schiff.

causes and make the differential diagnosis[33]. The ultrasonographic examination of the liver is a simple and useful procedure to have information about the dimension and the characteristics of the liver tissue[34]. In few cases, T1D patients were submitted to an abdomen computed tomography scan. Unfortunately, HG cannot be clinically distinguished from non-alcoholic fatty liver disease or non-alcoholic steatohepatitis (NASH) by history, physical examination or ultrasound: the gold standard examination is the liver biopsy[36]. The preparation of the tissue is very important for the identification of the glycogen in tissue sections. The Carnoy's solution is rapid acting, gives good nuclear preservation, retains glycogen and dissolves lipids[37]. The cytoplasmic swelling due to glycogen can be quickly demonstrated by the staining with Best's carmine or periodic acid-Schiff (PAS) with and without diastase since the slides treated with diastase, that digest the glycogen, lack the PAS positive staining[34]. The main histological features of HG are marked glycogen accumulation leading to pale swollen hepatocyte, no or mild fatty change, no or minimal inflammation, no or minimal spotty lobular necrosis, and intact architecture with no significant fibrosis[35]. Best's carmine is another common used stain for glycogen, that appears bright red in sections. On the contrary, in hematoxylin & eosin sections, pale hepatocytes loose their glycogen during tissue preparation and may give a hint to hepatic glycogenosis (Figure 2)[37].

Navigator-gated and gradient-echo shimmed point-resolved spectroscopy with proton hydrogen1 (1H) magnetic resonance (MR) has been recently proposed to quantify liver glycogen concentrations in vivo, even if this measurement is more challenging than just lipid quantification[38]. In previous studies, an MR technique was used with (1-^{13}C) glucose to measure changes in net hepatic glycogen concentration in normal and diabetic subjects[39,40].

To our best knowledge, in only one study the authors investigated the liver by the means of the MR imaging, with anatomical purposes[41].

Whereas it is well known that glycogen storage diseases, particularly type I , develop hepatic adenoma that potentially progress into hepatocellular carcinoma (HCC), to our best knowledge no data have been published about the association of diabetic glycogenosis and the progression of carcinogenesis to HCC[42-47].

TREATMENT

The more the T1D patients (and their caregivers) obtain a good glycemic control, the more HG is expected to be minimal.

The Diabetes Control and Complication Trial (DCCT) is a well-known multicenter randomized trial that compared intensive with conventional therapy in insulin-dependent diabetes mellitus, demonstrating a prevention of diabetic complications[48]. The percentage of adolescent (13-18 years old) was 9%-19% of 1441 patients, with a 2.6-8.9 years of disease duration, a starting insulin dose of 0.62-0.72 U/kg of body weight/day and an insulin dose after 5 year of 0.46-1.10 U/kg of body weight/day[48,49].

As it has been described in the literature, the mean insulin dose in T1D patients with HG was significantly higher than in DCCT trial (1.33 U/kg), having been treated with supra-physiologic doses of insulin (Table 1).

Repeated ketoacidosis episodes in T1D significantly increase the risk for hepatic glycogen overload, since diabetic ketoacidosis (a fatal complication of poor controlled diabetes) is usually treated with sustained levels of intravenous insulin (in the presence of high glucose blood concentrations). As matter of fact, a high percentage of the HG cases described in the literature presented diabetic ketoacidosis, with a frequency of about 40% (14/35 cases), confirming the association of sustained insulin treatment and the development of HG.

With a significant difference from NASH, HG is completely reversible with a good metabolic control[50,51]. Adequate management of glucose and insulin levels can result in complete remission of clinical, laboratory and histological abnormalities[52]. Continuous subcutaneous insulin infusion should be considered as an option because the insulin requirements usually come down with improved

glycemic control[41]. In severe and rare cases, pancreatic transplantation has been reported to be effective[53].

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P- Reviewer: Bannasch P, Pastromas S **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
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ISBN 978-0-9861420-0-0

Glycemic control indicators in patients with neonatal diabetes mellitus

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Received: November 26, 2013 Revised: January 24, 2014
Accepted: March 13, 2014
Published online: April 15, 2014

Abstract

Neonatal diabetes mellitus (NDM) is a type of diabetes mellitus caused by genetic abnormality which develops in insulin dependent state within 6 mo after birth. HbA1c is widely used in clinical practice for diabetes mellitus as the gold standard glycemic control indicator; however, fetal hemoglobin (HbF) is the main hemoglobin in neonates and so HbA1c cannot be used as a glycemic control indicator in NDM. Glycated albumin (GA), another glycemic control indicator, is not affected by HbF. We reported that GA can be used as a glycemic control indicator in NDM. However, it was later found that because of increased metabolism of albumin, GA shows an apparently lower level in relation to plasma glucose in NDM; measures to solve this problem were needed. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

Key words: Glycemic control; HbA1c; Glycated albumin; Fructosamine; 1,5-anhydroglucitol; Neonatal diabetes mellitus

Core tip: Neonatal diabetes mellitus (NDM) is a type of diabetes mellitus caused by genetic abnormality which develops in insulin dependent state within 6 mo after birth. Because fetal hemoglobin (HbF) is the main hemoglobin in neonates, HbA1c cannot be used as a glycemic control indicator in NDM. On the other hand, glycated albumin (GA), another glycemic control indicator, is not affected by HbF. We reported that GA can be used as a glycemic control indicator in NDM. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

Original sources: Suzuki S, Koga M. Glycemic control indicators in patients with neonatal diabetes mellitus. *World J Diabetes* 2014; 5(2): 198-208 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/198.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.198

INTRODUCTION

To prevent chronic diabetic complications, it is necessary to try to achieve normoglycemia as much as possible. Previously, glycemic control used to be evaluated by plasma glucose or urinary glucose. However, these indicators fluctuate continuously due to factors such as dietary intake, and it was difficult to evaluate glycemic control correctly by taking measurements at a particular time. Therefore, hemoglobin A1c (HbA1c), which reflects mean plasma glucose during the past 1 to 2 mo, was introduced as a glycemic control indicator[1], and is now widely used in clinical practice for diabetes mellitus. HbA1c can be used to evaluate glycemic control status; if poor glycemic control is observed, it is possible to make additions, changes, *etc.* to the treatment of diabetes mellitus[2].

Large-scale researches such as the Diabetes Control and Complications Trial revealed that HbA1c is related to the development and progression of diabetic microangiopathy[3]. That is, the development and progression of diabetic microangiopathy can be prevented by maintain-

ing excellent glycemic control using HbA1c as an indicator. Recently, it also became possible to use HbA1c for the diagnosis of diabetes mellitus[4].

However, the following problems of HbA1c were pointed out: (1) abnormal HbA1c values may be observed because of variantl hemoglobin, hemolytic anemia, *etc*.; (2) HbA1c does not correctly reflect short-term glycemic control status; and (3) HbA1c does not correctly reflect postprandial plasma glucose/fluctuation of plasma glucose. Accordingly, new glycemic control indicators such as fructosamine, 1,5-anhydroglucitol (1,5-AG), and glycated albumin (GA) were introduced. Although these indicators compensate the disadvantages of HbA1c, they have their own disadvantages. For example, 1,5-AG is affected by the threshold of urinary glucose excretion in the kidney, and fructosamine and GA are affected by albumin metabolism[5].

Because fetal hemoglobin (HbF) is the main hemoglobin in neonates, HbA1c cannot be used as a glycemic control indicator in neonates. Therefore, glycemic control in neonatal diabetes mellitus (NDM) was traditionally performed using blood glucose measured by self-monitoring of blood glucose as an indicator, without using a glycemic control indicator. We demonstrated that GA, which is not affected by HbF, reflects glycemic control in NDM and can be used as a glycemic control indicator in NDM[6]. We also obtained various other findings about GA and HbA1c in neonates/infants or NDM. In this review, we outlined the most recent findings concerning glycemic control indicators in neonates or NDM.

NEONATAL DIABETES MELLITUS

NDM is a type of diabetes mellitus caused by single-gene abnormality which develops acutely in insulin dependent state; NDM accounts for the majority of cases of diabetes mellitus which develops within 6 mo after birth[7]. The frequency of NDM according to this definition is 1 in 89000 births, showing that NDM is a rare disease[8]. So far, more than 20 causative genes of NDM have been discovered; genetic mutations of some kind have been identified in not less than 70% of patients[8,9]. NDM is similar to type 1 diabetes mellitus in terms of the form of development (diabetes mellitus develops acutely); however, type 1 diabetes mellitus very rarely develops within 6 mo after birth, judging from studies on the frequency of human leukocyte antigen risk alleles and the presence of pancreatic autoantibodies[10,11]. Based on the clinical course, NDM is classified into two major categories: transient NDM (TNDM) and permanent NDM (PNDM)[12]. TNDM is a condition in which insulin secretion is restored spontaneously and normoglycemia is achieved without treatment; PNDM is a condition in which remission is not achieved and life-long treatment is required. The frequency of TNDM is about 60%, and that of PNDM is about 40%.

Although patients with TNDM require insulin therapy at the time of onset because of marked hyperglycemia, they can be weaned from insulin therapy at an average of 3 mo after the start of treatment[13-16]. This is called the remission period. However, in about half of patients, diabetes mellitus relapses from childhood to adolescence[14,17]. In 70% of patients with TNDM, the cause is overexpression of an imprinted gene *PLAGL1* which is located in the chromosome 6q24 region and is expressed from paternal allele (6q24-TNDM)[14,15,18,19]. In 25% of patients with TNDM, mutations of *KCNJ11* and *ABCC8* genes which encode the ATP-sensitive potassium channel (K_{ATP} channel) essential for glucose-stimulated insulin secretion have been identified (K_{ATP}-TNDM)[14,20,21]. 6q24-TNDM has the following characteristics: (1) it often develops within 1 wk after birth; (2) it is often diagnosed asymptomatically on routine blood collection; and (3) it is rarely accompanied by ketoacidosis[15,19]. On the other hand, the time of diagnosis of K_{ATP}-TNDM is 1 to 4 mo after birth, which is later than that of 6q24-TNDM[14].

The main causes of PNDM are K_{ATP} channel abnormality [*KCNJ11* gene (31%); *ABCC8* gene (10%)] and insulin gene mutations (12%); the median age at the time of diagnosis is 8 wk after birth and 10 wk after birth, respectively[9]. In contrast to TNDM, PNDM shows symptoms such as dehydration, poor sucking, and poor weight gain at the time of onset and is often accompanied by ketoacidosis[15,19]. A large proportion of other causative genes are expressed by autosomal recessive inheritance and account for about 10% of PNDM. In about 35% of patients with NDM, causative genes have not been identified[7].

Insulin therapy is required at the time of onset of NDM regardless of disease type in order to improve metabolic abnormality and weight increase[22]. It has been reported that because neonates have a small body and then receive a small dose of insulin, excellent glycemic control is achieved by an insulin pump which is capable of fine regulation[23-25]. As a treatment after withdrawal from the acute phase, a switch to high-dose administration of sulfonylurea (SU) drugs is an effective causal therapy for K_{ATP} channel abnormality; in not less than 90% of patients, a dramatic improvement of glycemic control is observed immediately without hypoglycemia and is maintained for a long period[26-28]. Therefore, when NDM is diagnosed, it is important to determine by gene analysis whether or not K_{ATP} channel abnormality is present. Early diagnosis makes it possible to switch to SU drugs during infancy, resulting in an extremely high quality-of-life[29-32].

1,5-ANHYDROGLUCITOL IN NEONATES

1,5-AG is a polyol with a structure in which hydroxyl at the 1st position of glucose is reduced; 1,5-AG is contained in a wide variety of food, but is hardly metabolized in the body[33]. Therefore, after being absorbed from the intestine, 1,5-AG contained in food is widely distributed in various organs to form an internal pool. The amount of 1,5-AG supplied from daily food intake is smaller than the internal pool, and so there is no change in serum 1,5-AG concentration before and after meal. Excessive intake of 1,5-AG is excreted in urine.

Usually, about 180 g of glucose is excreted daily from glomeruli; about 100% of the excreted glucose is reabsorbed by sodium glucose cotransporter 2 (SGLT2), which is located in proximal renal tubules and are specific to glucose[34], and SGTL1, which is located downstream of SGLT2. After the onset of diabetes mellitus, excretion of glucose will increase; when the increased excretion of glucose exceeds the reabsorption capacity of SGLT2 and SGLT1, reabsorption of glucose *via* 1,5-AG/mannose/fructose cotransporter (SGLT4), which is located downstream of SGLT2 and SGLT1, will start. Because glucose is usually not present, 99.9% of 1,5-AG is reabsorbed by SGLT4; however, this reabsorption mechanism is common to glucose; therefore, if inflow of glucose into tubules increases, reabsorption of 1,5-AG will be inhibited[35-37]. Therefore, in a hyperglycemic condition, excretion of 1,5-AG into urine will increase and serum 1,5-AG will decrease. Thus, serum 1,5-AG is a glycemic control indicator which reflects the degree of urinary glucose excretion.

Because serum 1,5-AG increases and decreases by excretion of urinary glucose, serum 1,5-AG reflects short-term changes in glycemic control more subtly than HbA1c. When glycemic control has worsened rapidly, serum 1,5-AG will decrease rapidly because the increased excretion of a large amount of glucose will inhibit reabsorption of 1,5-AG *via* SGLT4. In patients with marked hyperglycemia and a high excretion of urinary glucose, serum 1,5-AG will not increase in a short period even if glycemic control has improved rapidly because the internal pool of 1,5-AG has decreased.

Serum 1,5-AG is also affected by the threshold for urinary glucose excretion, and therefore shows a low level in renal glycosuria in which the threshold decreases. In addition, serum 1,5-AG shows an abnormally low level in conditions such as chronic renal failure in which reabsorption of 1,5-AG decreases[38-40], pregnancy[41], oxyhyperglycemia in which urinary glucose is observed transiently[42], patients receiving long-term hyperalimentation[43], and liver cirrhosis[44,45]. One of the causes of an abnormally high level of 1,5-AG is oral administration of a kind of Chinese medicines such as Ninjin-yoei-to and Kami-kihi-to which contain large amounts of 1,5-AG[46].

It is known that serum 1,5-AG during the neonatal period shows an apparently low level[47]. This is considered to be due to a small intake of 1,5-AG during the neonatal period. We reported that serum 1,5-AG is significantly lower in subjects with a habit of consuming dairy products than in subjects without such a habit[48]. The fact that breast milk or formula which contains galactose is the main source of nutrition during the neonatal period may be related to a low level of serum 1,5-AG in neonates.

FRUCTOSAMINE IN NEONATES

Protein undergoes glycation reaction in accordance with plasma glucose concentration, and ketoamine, an early Maillard reaction product, is produced *via* aldimine. Because the side chain binding of ketoamine takes a fructose structure, ketoamine is generically named fructosamine. Fructosamine is measured using the property that fructose-lysine (fructosamine), in which glucose is bound to the lysine residues of protein, has reducing ability under alkaline conditions. A large proportion of measurements are made by the chemical method; measurements are made by colorimetric determination by producing reduction color reaction using nitroblue tetrazolium (NBT) as a chromogen. Because 60% to 70% of serum protein is albumin, the main component of fructosamine is glycated albumin, but fructosamine contains glycated lipoprotein and glycated globulin as well. Fructosamine is not affected by anemia or variant hemoglobin. In addition, because the turnover of albumin, which accounts for the most part of serum protein, is faster than that of hemoglobin, it is possible to evaluate short-term glycemic control by measuring fructosamine[49]. A low fructosamine level is observed in hyperthyroidism[50,51] and nephrotic syndrome[52] in which protein (albumin) metabolism is accelerated; a high fructosamine level is observed in hypothyroidism[50,51] in which protein (albumin) metabolism is prolonged.

HbA1c and GA are glycation products of hemoglobin and albumin (single proteins), respectively, whereas fructosamine is the generic name of all glycated proteins and lacks specificity. Because albumin accounts for 60% to 70% of serum protein, fructosamine has similar properties to GA; however, there is a problem that because other glycated proteins are measured as well, a high fructosamine level is observed in myeloma[53]. Because HbA1c and GA are expressed as the ratio of hemoglobin and the ratio of albumin, respectively, they are not affected by dilution of serum; on the other hand, because fructosamine is expressed as reducing ability per 1 mL of serum, it is affected by serum protein concentration, and an apparently low level of fructosamine is observed in dilutional anemia. The level of fructosamine in young children is lower than that in adults[54], which is also partly due to low serum protein concentration. Because fructosamine is measured by colorimetric determination based on reduction color reaction, fructosamine is affected by bilirubin with reducing ability, *etc*. It is considered that the effects of ascorbic acid and vitamin E are mild; however, if a large amount of ascorbic acid or vitamin E is consumed, measurement of fructosamine may be affected.

GLYCEMIC CONTROL INDICATORS OF CORD BLOOD

The composition of hemoglobin in healthy adults is as follows: adult hemoglobin (HbA): 97%; HbA2: 2.5%; HbF: 0.5%[55]. On the other hand, HbF accounts for 80% to 90%, and HbA accounts for only 10% to 20% immediately after birth. After then, HbF decreases logarithmically and is replaced by HbA; by 6 mo after birth, the largest proportion of Hb is HbA; however, it is not until 1 year after birth when the proportion of HbF decreases to less than 1% (level of HbF in adults)[56,57]. Therefore, it

is difficult to use the cation exchange high-performance liquid chromatography (HPLC) method, the immunological (latex immunoturbidimetry; LA) method, and the enzyme method which specifically measure HbA1c as glycemic control indicators in NDM.

We measured glycohemoglobin (GHb) in cord blood by various methods[58]. GHb measured by the HPLC method was less than the detection limit when Arkray's HA-8180 was used and was as low as 1.8% ± 0.2% when Tosoh's G8 was used. GHb measured by the LA method was less than the detection limit; HbA1c measured by the enzyme method was 1.1% ± 0.3%. Because these methods for measuring GHb measure HbA1c specifically and do not measure glycated HbF, the result is less than sensitivity or a very low level, and it was confirmed that these methods cannot be used as glycemic control indicators in NDM.

It is considered that measurement of GHb by the affinity method using boronic acid may be used as a glycemic control indicator during the neonatal period as well because it measures all glycated hemoglobins[59,60]. It has been reported that GHb in cord blood is higher in patients whose mother has diabetes mellitus than in patients whose mother does not have diabetes mellitus[61-63]. Our investigation revealed that GHb was 3.9% ± 0.2%, which was slightly lower than the reference value for adults (4.6% to 6.2%)[58]. Plasma glucose in cord blood was normal (94 ± 27 mg/dL); therefore, it is considered that the low GHb levels were due to shortened life span of red blood cells[64].

GA in cord blood was 9.4% ± 1.1%, which was slightly lower than the reference value for adults (11.6% to 16.2%)[58]. We demonstrated that low GA levels are observed in neonates because albumin metabolism in neonates is accelerated[65,66]. Low GA levels in cord blood are considered to be due to accelerated metabolism of albumin.

The level of 1,5-AG in cord blood measured in pregnant women including those with diabetes mellitus was similar to that in maternal blood at the time of delivery[67]. This finding was considered to be due to the fact that 1,5-AG in maternal blood was distributed in the fetus *via* the placenta.

The above results show that both GHb measured by the affinity method and GA were slightly lower than the reference value for adults, but could be used as glycemic control indicators in NDM. On the other hand, HbA1c measured by the HPLC method, the LA method, or the enzyme method and 1,5-AG cannot be used as glycemic control indicators.

GLYCEMIC CONTROL INDICATORS IN NDM: HBA1C AND GA

The etiologic diagnosis and treatment of NDM have been making rapid progress; however, there have been few studies on glycemic control indicators useful for evaluating the diagnosis of NDM and effects of therapy. Therefore, we hypothesized that GA is a useful glycemic control indicator in NDM[58] and conducted an investigation[6]. We found that GA, as a glycemic control indicator in NDM, has various advantages: (1) GA is not affected by HbF; (2) unlike fructosamine, GA is not affected by serum protein (albumin) because it is expressed as a ratio to albumin; (3) unlike fructosamine, GA is not affected by other proteins and has a high specificity because it reflects glycation products of a single protein (albumin); (4) GA reflects plasma glucose during a shorter period than HbA1c; and (5) HbA1c reflects mean plasma glucose, whereas GA reflects fluctuation of plasma glucose (postprandial hyperglycemia) in addition to mean plasma glucose[68-70]. HbA1c (%) is expressed as HbA1c/total Hb; therefore, if HbF is high, a relatively low HbA1c level will be observed. At the time of onset of NDM (mostly 1 to 2 mo after birth), a large amount of HbF remains in blood; therefore, a lower HbA1c level is observed in relation to plasma glucose level. In addition, it is estimated that during infancy, during which HbA increases, if plasma glucose level is constant, HbA1c will increase. In fact, in an investigation of five patients with NDM (age at the time of diagnosis: 38 ± 20 d), plasma glucose was markedly high [29.7 ± 13.1 mmol/L (535 ± 236 mg/dL)], whereas HbA1c measured by the HPLC method was within the normal range (5.4% ± 2.6%)[6]. As the course of treatment progressed, plasma glucose tended to decrease (Figure 1A), whereas HbA1c tended to increase (Figure 1B). A significant negative correlation was observed between HbA1c and HbF (Figure 2A), whereas no significant correlation was observed between HbA1c and plasma glucose level (Figure 2B). On the other hand, GA at the time of diagnosis was abnormally high (33.3% ± 6.9%)[6]. In contrast to HbA1c, GA decreased as treatment progressed (Figure 1C) and showed a strong positive correlation with plasma glucose level (Figure 2C). Thus, it was found that GA, but not HbA1c, is an appropriate glycemic control indicator in NDM.

From what age can HbA1c be used as a glycemic control indicator? Alternatively, if the effect of HbF is excluded or if a different principle of measurement is employed, might HbA1c be an appropriate indicator? And when using GA as a glycemic control indicator in NDM, what should be taken into account? In the following chapters, we will discuss these issues in relation to the current status and challenges in infants and NDM.

HBA1C IN NEONATES AND NDM

As mentioned above, when HbA1c is expressed as HbA1c/total Hb, it cannot be used as a glycemic control indicator in NDM. There are two ways to eliminate the effect of HbF. One way is to determine the HbA1c level corrected by HbF (HbF corrected HbA1c) by the formula: HbA1c/(total Hb-HbF), resulting in the correction of an apparently low HbA level. The other way is to determine GHb relative to all hemoglobins including HbF and to use this as a glycemic control indicator. For the latter, it is possible to measure all GHb by the affinity method[71].

We measured HbA1c by the HPLC method and the

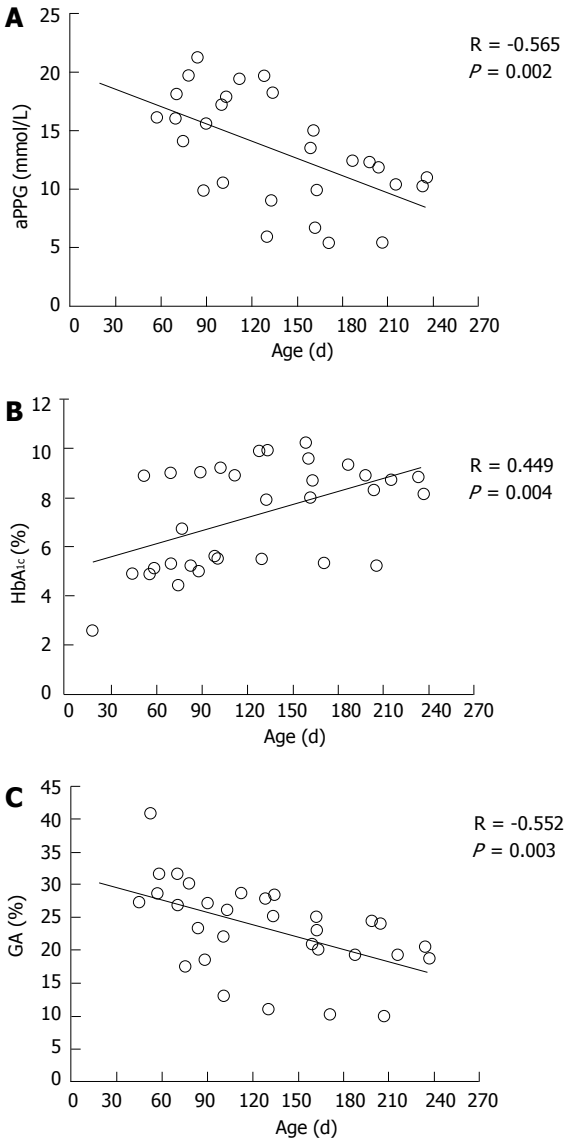

Figure 1 Time course of average preprandial plasma glucose for 1 mo (A), HbA1c (B), and glycated albumin (C) according to treatment in 5 patients with neonatal diabetes mellitus (modified from Ref[6], with permission from Copyright Clearance Center Inc.).

LA method in 26 healthy infants (0 to 8 mo old), calculated HbA1c values corrected by HbF [Adj-HbA1c (HPLC) and Adj-HbA1c (LA), respectively], measured GHb by the affinity method [GHb (Affinity)], and evaluated correlations between these values and plasma glucose and between these values and GA[72]. As a result, only GHb (Affinity) had a significant correlation with both plasma glucose and GA (Figure 3A). Adj-HbA1c (LA) was correlated only with GA (Figure 3B); Adj-HbA1c (HPLC) was not correlated with either plasma glucose or GA (Figure 3C). These results suggest that GHb (Affinity) may be used as a glycemic control indicator in NDM. In this research, however, GHb (Affinity) within one month was lower than the reference range of HbA1c during 8 to 12 mo (4.8% to 6.0%)[73], and a large proportion of GHb values from 1 to 5 mo were lower than the reference range. The following three factors are thought to contribute together to this finding. The first factor is the effect of a low plasma glucose level during infancy, especially within one month after birth[65,74]. The second factor is the short half-life of red blood cells (about 90 d) during infancy[64]. The third factor is the glycation rate of HbF which is considered to be lower than that of HbA. In this regard, Little *et al*[75] reported that GHb measured by the affinity method is low when a sample which contains not less than 15% of HbF is used. In the LA method, HbA1c is measured using antibodies which specifically recognize peptides including glycated valine of hemoglobin β-chain N-terminal[76]. Theoretically, when interpreting Adj-HbA1c (LA) levels, it is necessary to consider a low plasma glucose level and shortened half-life of red blood cells of the infant; however, it is considered that Adj-HbA1c (LA) may be used as a glycemic control indicator; in fact, a correlation between Adj-HbA1c (LA) and GA was observed. However, the LA method is too complicated to be used in clinical settings because it is necessary to measure HbF using the HPLC method. In addition, our investigation revealed that Adj-HbA1c (HPLC) is not an appropriate indicator for the evaluation of HbA1c in infants. In the HPLC analysis, HbF and HbA1c migrate to adjacent locations. When a high HbF level is observed, separation of HbF and HbA1c becomes insufficient and so HbA1c cannot be measured correctly, which is considered to be one of the causes of the above-mentioned phenomenon. On the other hand, Little *et al*[75] and Rohlfing *et al*[77] reported on HbF-corrected HbA1c as follows: if HbF is not more than 30%, HbA1c measured by the HPLC method using Tosoh's G7 and G8 can be used as a glycemic control indicator. However, they did not use samples which contained 30% or more of HbF, and they did not state whether or not Hb in the samples used was derived from infants; therefore, these facts may be the reason for the difference from our data obtained from samples of infants.

So far, there have been no studies on the age at which HbA1c can be used for patients with NDM, and so research is needed to clarify the relationship between mean plasma glucose and HbA1c and between CGM and HbA1c. Regarding the reference value of HbA1c in healthy infants, there is only a report by Jansen *et al*[73] who investigated 100 healthy infants of 8 to 12 mo old. In that report, the reference value of HbA1c for infants was 4.8% to 6.0%, which was similar to the reference value of HbA1c for adults (4.6% to 6.2%). From our results, HbA1c levels in most infants of 6 mo of age or older were also within the reference range shown by Jansen *et al*[73] HbF decreases to less than 5% by 6 mo after birth[56,57]; therefore, it is considered possible to use HbA1c as a glycemic control indicator in patients with NDM of 6 mo of age or older.

GA IN NEONATES AND NDM

GA is a useful glycemic control indicator under conditions in which hemoglobin metabolism is affected. On the other hand, abnormal albumin metabolism affects GA. It has been reported under various conditions that

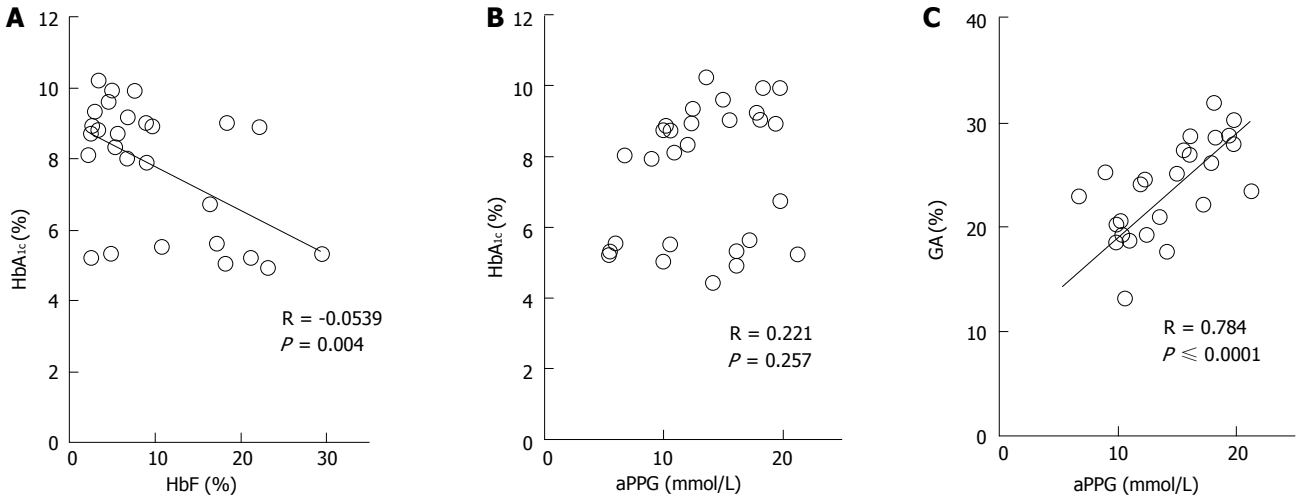

Figure 2 Correlations between HbA1c and HbF (A) and between HbA1c and average preprandial plasma glucose for 1 mo (B) and correlation between glycated albumin and average preprandial plasma glucose in 5 patients with neonatal diabetes mellitus (C) (modified from Ref[6], with permission from Copyright Clearance Center Inc.).

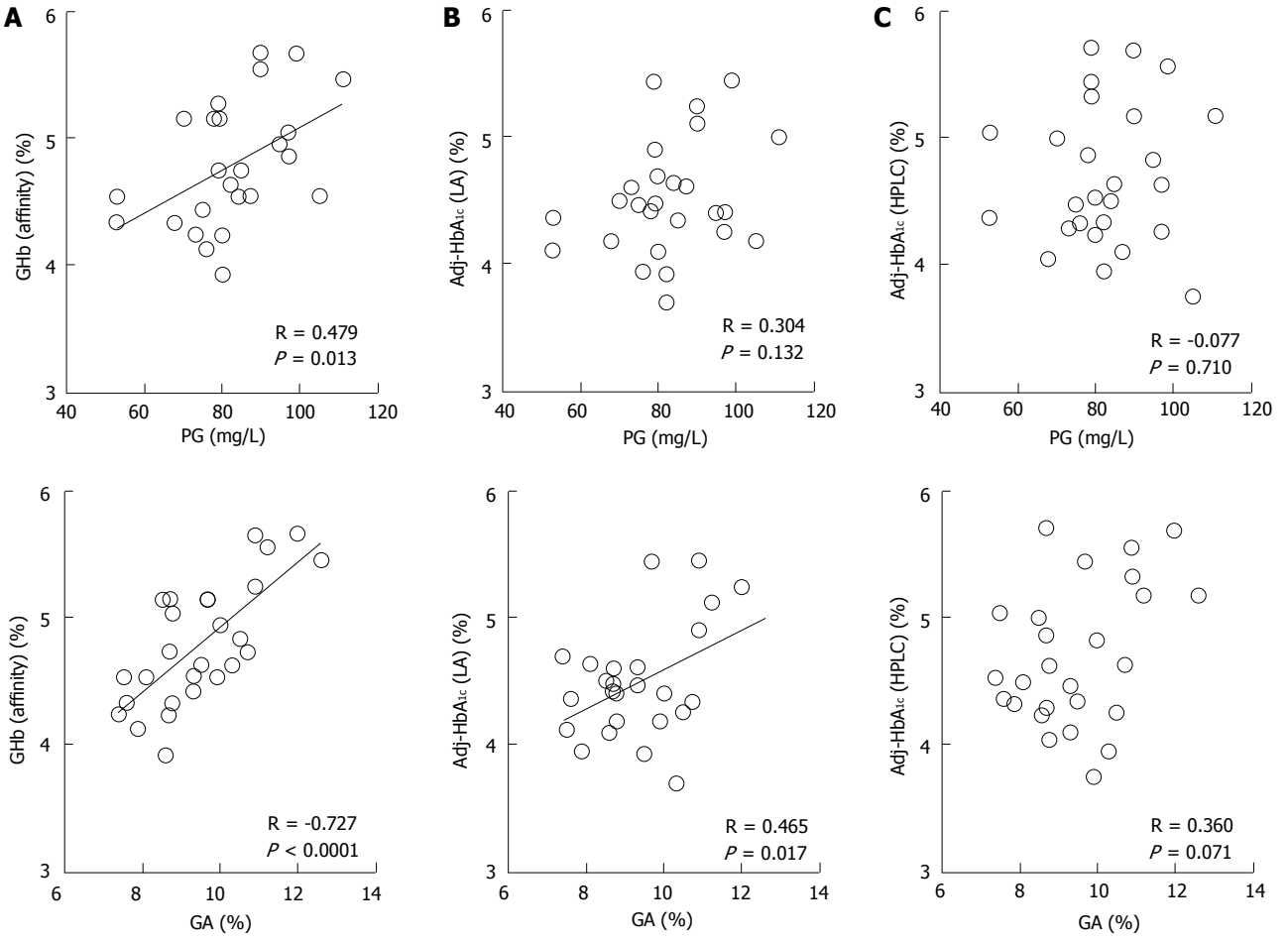

Figure 3 Correlations between glycated hemoglobin measure by various methods and plasma glucose or glycated albumin. Correlations between GHb measured by the affinity method [GHb (affinity)] (A), HbF-adjusted HbA1c measured by the immunological method [Adj-HbA1c (LA)] (B), and HbF-adjusted HbA1c measured by the HPLC method [Adj-HbA1c (HPLC)] (C), and PG or GA in 26 healthy infants were shown (modified from Ref[72], with permission from Copyright Clearance Center Inc.). GA: Glycated albumin; PG: Plasma glucose; GHb: Glycated hemoglobin; HbF: Fetal hemoglobin.

GA shows a low level when albumin metabolism is accelerated and shows a high level when albumin metabolism is suppressed[78].

While GA is a useful glycemic control indicator in patients with NDM, it is necessary to keep in mind the following characteristics of GA during infancy: (1) it

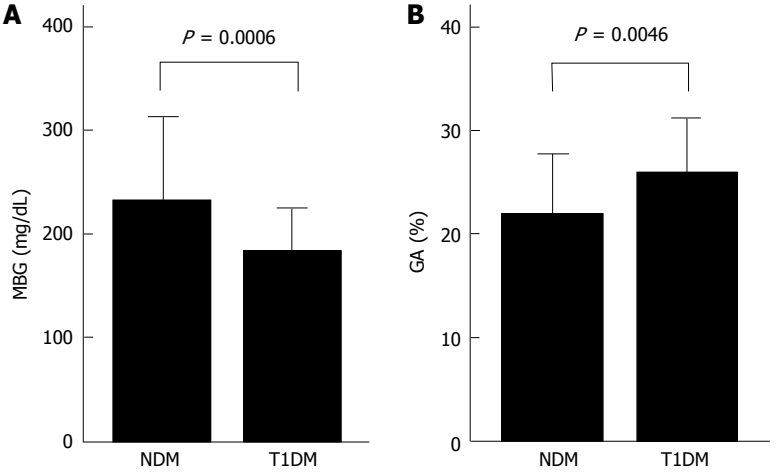

Figure 4 Comparisons of mean blood glucose for 1 mo (A) and GA (B) in 6 patients with neonatal diabetes mellitus and in 18 patients with type 1 diabetes mellitus (modified from Reference[65], with permission from Copyright Clearance Center Inc.).

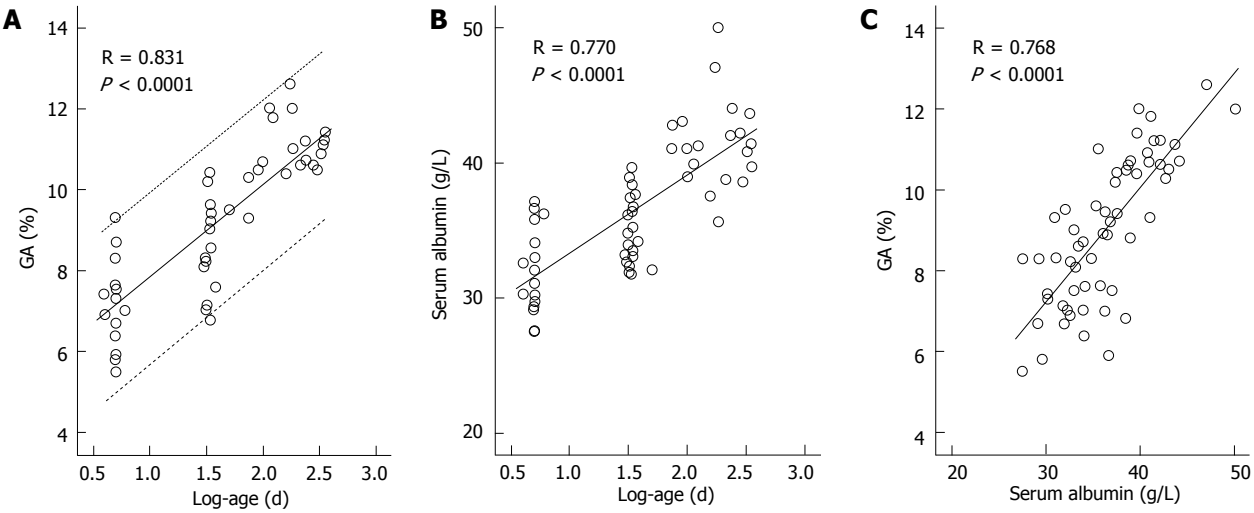

Figure 5 Correlations between glycated albumin and age and between serum albumin and age and correlation between glycated albumin and serum albumin in healthy infants. A: Correlation between glycated albumin (GA) and log-age. The dotted line shows the 95%CI; B: Correlation between serum albumin and log-age; C: Correlation between GA and serum albumin (modified from Ref[66], with permission from Copyright Clearance Center Inc.).

shows a lower level in relation to plasma glucose; and (2) it shows a positive correlation with logarithmically transformed age[65,66]. For GA in healthy infants, before the currently widely used enzyme method was developed[79], it had already been reported that GA measured by the HPLC method was lower than the reference value for adults[54]. It is known that protein metabolism is accelerated during infancy[80,81]. In addition, it has been reported that albumin synthesis is accelerated as well[82]. Therefore, acceleration of albumin metabolism may contribute to a low GA level during infancy. We compared the relationship between GA and plasma glucose level in patients with NDM and in patients with juvenile type 1 diabetes mellitus (T1DM), and found that patients with NDM had higher plasma glucose levels but lower GA levels than patients with T1DM (Figure 4); thus, we obtained a result which supports the phenomenon of accelerated metabolism of albumin during infancy[65]. In addition, we investigated in healthy infants the relationship between change in GA according to age and plasma glucose and between change in GA according to age and serum albumin. As a result, a strong positive correlation was observed between GA and logarithmically transformed age in days (Figure 5A), and multivariate analysis revealed that age and serum albumin affect GA levels more significantly than plasma glucose[66]. Because GA is expressed as a percentage relative to serum albumin, it is not affected by serum albumin, which is an advantage of GA over fructosamine[54]. However, an increase in serum albumin associated with aging is observed during infancy (Figure 5B) and there is a positive correlation between GA and serum albumin during this period (Figure 5C)[66]. Accordingly, we determined the reference value of GA in infants according to age in mo from the regression equation of GA and age, and proposed that a comparison between GA level and the reference value[65].

On the other hand, we found that regardless of age, GA can be evaluated based on the reference value for adults without using the reference value for infants by determining age adjusted GA (Aa-GA)[83]. We investigated GA in 376 subjects without diabetes mellitus of a wide range of age (neonates, children, and adults), and found

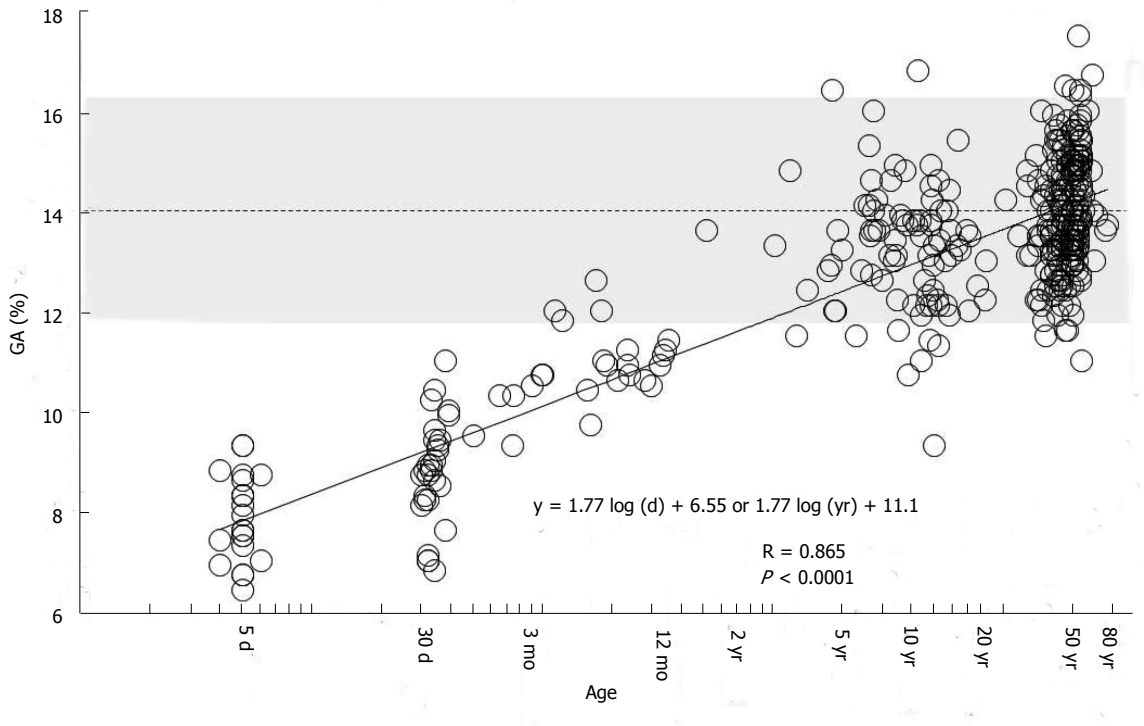

Figure 6 Correlation between glycated albumin and age in days (logarithmic transformation) in 376 healthy subjects (age: 4 d to 78 years). The dotted line indicates the mean reference value for adults (14%), and the shading indicates the range of reference values for adults (11.7% to 16.2%) (modified from Ref[83], with permission from Royal Society of Medicine).

that GA can be expressed as a primary regression equation of logarithmically transformed age (Figure 6). Based on this equation, the following formula for calculating Aa-GA was derived: Aa-GA = GA × 14.0 /[1.77 × log-age (d) + 6.55] or Aa-GA = GA × 14.0 /[1.77 × log-age (yr) + 11.1]. As mentioned above, GA in NDM shows an apparently low level; therefore, if GA in NDM is compared with the reference value for adults, the glycemic control status may be underestimated. By calculating Aa-GA and comparing it with the reference value for adults, it is possible to accurately evaluate the glycemic control status in NDM. The advantages of evaluating Aa-GA by the reference value for adults instead of evaluating GA by the reference value for infants according to age in month are as follows: (1) it is not necessary to consider the reference value according to age in month; and (2) regardless of age, it is possible to make comparisons of longitudinal changes in glycemic control status.

It is known that because the half-life of GA is shorter than that of HbA1c, GA reflects short-term plasma glucose correctly[84,85]. This characteristic also indicates the usefulness of GA as a glycemic control indicator in NDM. Because a large proportion of NDM develops within one month, the duration of the hyperglycemic status is short. This form of development is similar to that of fulminant type 1 diabetes mellitus[86]. In fulminant type 1 diabetes mellitus, pancreatic beta cells are destroyed in a very short period, and ketoacidosis develops shortly after the onset of diabetic symptoms. Therefore, at the time of onset, HbA1c is normal or only slightly high, but GA is already obviously high[87]. We reported that GA at the time of onset of NDM was abnormally high (33.6 ± 6.9%) in all patients[6], and an abnormally high GA level in NDM may be useful for differential diagnosis from transient hyperglycemia. In addition, when evaluating remission of patients with TNDM and when evaluating the effect of SU drugs administered to patients with PNDM, it will be possible to promptly evaluate an improvement of such glycemic control by using GA[5].

CONCLUSION

The usefulness of GA as a glycemic control indicator in NDM was demonstrated. However, it was found that GA is affected by albumin metabolism and shows an apparently low level. Therefore, it is necessary to compare GA with the reference value according to age or to calculate age-adjusted GA (Aa-GA). On the other hand, HbA1c measured by the HPLC method, the LA method, or the enzyme method does not correctly reflect the glycemic control status because it is affected by a high HbF level. GHb measured by the affinity method reflects the glycemic control status in NDM; however, this method is currently hardly used and cannot easily measure GHb routinely. In addition, it is unknown whether the kinetics of glycation reaction of HbF are similar to those of HbA. Taking into account such circumstances, it is desirable to select GA as a glycemic control indicator for patients with NDM and to evaluate the glycemic control status using Aa-GA.

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P- Reviewers: Bagdure D, Izawa KP, Tamemoto H
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Insulin plus incretin: A glucose-lowering strategy for type 2-diabetes

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Received: October 14, 2013 Revised: November 25, 2013
Accepted: December 12, 2013
Published online: February 15, 2014

Abstract

There are many advantages of combining incretin therapy [glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors] with insulin therapy as a glucose-lowering strategy in type 2 diabetes. One important advantage is the complementary mode of the mechanistic action of incretin and insulin therapy. Another advantage is the reduction in risk of hypoglycemia and weight gain when adding incretin therapy to insulin. Several clinical trials have studied the addition of GLP-1 receptor agonists [exenatide BID (twice daily), lixisenatide, albiglutide] or DPP-4 inhibitors (vildagliptin, sitagliptin, saxagliptin, alogliptin, linagliptin) to ongoing insulin therapy or adding insulin to ongoing therapy with a GLP-1 receptor agonist (liraglutide). These studies show improved glycemia in the presence of limited risk for hypoglycemia and weight gain with the combination of incretin therapy with insulin. This article reviews the background and clinical studies on this combination.

Key words: Type 2 diabetes; Glucose lowering; Insulin therapy; Glucagon-like peptide-1 receptor agonists; Dipeptidyl peptidase-4 inhibitors; Incretin therapy; Combination

Core tip: Incretin therapy (glucagon-like peptide-1 receptor agonists or dipeptidyl peptidase-4 inhibitors) combined with insulin therapy is a glucose-lowering strategy in type 2 diabetes. The combination allows a complementary mode of mechanistic action and, as demonstrated in several clinical trials, is glucose-lowering in association with limited risk for hypoglycemia and weight gain. The combination is a promising strategy in patients in whom metformin with either incretin therapy or basal insulin is insufficient for adequate glycemic control. This article reviews the background and clinical studies on this combination.

Original sources: Ahrén B. Insulin plus incretin: A glucose-lowering strategy for type 2-diabetes. *World J Diabetes* 2014; 5(1): 40-51 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i1/40.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i1.40

INSULIN IN COMBINATION WITH INCRETINS: A MORE COMMONLY USED GLUCOSE-LOWERING THERAPY

Life style changes accompanied by addition of metformin are often first line glucose reducing therapy in type 2 diabetes[1,2]. When metformin as the only pharmaceutical agent is insufficient for adequate glycemic control, several options are currently available. Of these, sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists and insulin were recently suggested by the joint position statement from the American Diabetes Association and the European Association of the Study of Diabetes to be potentials as an add-on to metformin[1]. They were suggested to be individualized to target the best combination for the individual patient. However, even after combination of metformin with any of these second-line therapies, many patients still do not reach the glycemic target which is mainly due to the progression of the disease. At this stage, three-drug combinations are suggested to be

used, involving metformin in combination with two of the other options. One such three-drug combination is the combination of insulin therapy with incretin therapy (+ metformin) as a glucose-reducing strategy of type 2 diabetes[2-6]. This article reviews the current evidence and experience for this combination.

BASIS FOR INCRETIN THERAPY

Incretin therapy is based on the anti-diabetic effects of GLP-1[7]. As an incretin hormone, GLP-1 is released from the gut after meal ingestion and augments insulin secretion in a glucose-dependent manner[7,8]. This effect on the beta cells is achieved through activating specific GLP-1 receptors, which are G protein coupled receptors[9]. GLP-1 also has an important effect to inhibit glucagon secretion[10]. These double effects on islet hormone secretion are of importance for the anti-diabetic action of incretin therapy and, furthermore, by targeting the double alpha and beta cell dysfunction, incretin therapy targets a main pathophysiological cause of the disease[11]. GLP-1 receptors are, however, also expressed in other cells and therefore GLP-1 also exhibits extra-islet effects, such as delay of gastric emptying[12] and satiety through a central effect in the hypothalamus[13]. GLP-1 also has the potential of preserving beta cell function through inhibition of apoptosis[14], although this has so far only been demonstrated in animal studies and not shown in humans.

The first study showing an anti-diabetic action of GLP-1 was published in 1992[15]. In the early development of GLP-1 as a therapy, GLP-1 had to be given as an intravenous infusion since the hormone is rapidly inactivated by DPP-4[16]. The two successful strategies for incretin therapy used this knowledge and today we have several GLP-1 receptor agonists which are not or only weakly inactivated by DPP-4 and DPP-4 inhibitors[17-20].

GLP-1 receptor agonists are injected subcutaneously once or twice daily [exenatide BID (twice daily), liraglutide, lixisenatide] or once weekly {exenatide once weekly [Quaque weekly (QW)]}. In addition, once weekly GLP-1 receptor agonists are in late clinical development (albiglutide, semaglutide, dulaglutide)[17,20]. The GLP-1 receptor agonists therefore differ in several respects, such as dosage regimen. However, GLP-1 receptor agonists also differ in other aspects, as was recently reviewed[17-20]. Thus, the different GLP-1 receptor agonists have different molecular structures and in this context, they may be derived from exendin-4, showing approximately 50% homology with native GLP-1 (exenatide, lixisenatide), or they may be true GLP-1 analogues with a structure showing a high (> 90%) homology to GLP-1 (liraglutide, albiglutide, semaglutide, dulaglutide). The GLP-1 receptor agonists also differ in molecular size since they may be similar in size to native GLP-1 (exenatide, lixisenatide, liraglutide, semaglutide) or be 15-20 times bigger because of fusion of GLP-1 with albumin (albiglutide) or immunoglobulin (dulaglutide).

DPP-4 inhibitors are oral agents given once or twice daily (sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin, tenelagliptin, anagliptin, gemagliptin)[18,19]. They are different from each other in terms of molecular structure, although they are all small molecules, and they also differ, besides in pharmacokinetics with relevance for dosing regimen, in elimination mechanisms, as was recently reviewed[21].

Incretin therapy is today established as an add-on treatment to metformin and is also used in other conditions; it results in reduction of both fasting and postprandial glucose and it is associated with a low risk of hypoglycemia and no weight gain (weight reduction or weight neutrality)[19,20,22].

RATIONALE FOR COMBINATION INSULIN THERAPY PLUS INCRETIN THERAPY

The combination of incretin therapy and insulin therapy was initially not clearly evident during the development of incretin therapy. Instead, incretin therapy was mainly developed for combination with oral antihyperglycemic agents, in particular metformin. This is still a very important combination. However, as discussed for GLP-1 receptor agonists[4] and DPP-4 inhibitors[2,3], incretin therapy offers mechanistic advantages when used in association with insulin, which makes this combination a promising strategy for treatment.

The mechanistic complementary actions of the two approaches relate to reduction in fasting glucose, reduction in postprandial glucose, the low risk for hypoglycemia and the prevention of weight gain. More mechanistic studies are required, however, for a full appreciation of the complementary actions of insulin and incretins in combination.

Fasting glucose

Reduction of fasting glucose is a major goal for glucose-lowering therapy since fasting glucose contributes largely to hemoglobin A1c (HbA1c)[23,24]. A main effect of basal insulin is the reduction of fasting glucose, which is achieved through increased peripheral (mainly muscle and fat tissue) glucose utilization and inhibited hepatic glucose output[25,26]. Also, GLP-1-receptor agonists and DPP-4 inhibitors reduce fasting glucose but this is achieved through other mechanisms than insulin; mainly a glucose-dependent inhibition of glucagon secretion from the islet alpha cells[10,27]. In addition, direct liver effects of GLP-1 may also contribute[28]. Hence, the combination of insulin with incretin therapy would be expected to complement each other to reduce fasting glucose.

Postprandial glucose

Postprandial glucose also contributes to HbA1c and is therefore a target for glucose-lowering therapy[23,24]. Postprandial glucose is mainly regulated by gastric emptying and the meal-induced islet hormone responses[29-31]. These effects are not appreciably affected by basal insulin. In contrast, incretin therapy reduces postprandial glucose,

although the mode of action to achieve this effect differs between GLP-1 receptor agonists and DPP-4 inhibitors. GLP-1 receptor agonists reduce postprandial glucose mainly by delaying gastric emptying[29-31]. This effect of GLP-1 shows, however, tachyphylaxis, meaning that during long-term and continuous stimulation, the effect is reduced[32,33]. Consequently, intermittently acting GLP-1 receptor agonists (exenatide BID, lixisenatide) have been shown to be more potent to reduce gastric emptying than continuously acting GLP-1 receptor agonists (liraglutide, exenatide QW)[34,35]. In contrast, DPP-4 inhibitors do not inhibit gastric emptying[36] but instead reduce postprandial glucose mainly through inhibiting postprandial glucagon levels and stimulating beta cell function[27,37]. Both incretin therapy strategies therefore reduce postprandial glucose and thus complement the lack of such an effect by insulin in the combination therapy.

Hypoglycemia

Hypoglycemia is an adverse event for glucose-lowering therapy and is occasionally the limitation factor for achieving good glycemic control. Hypoglycemia is associated with negative impact, such as unpleasant and sometimes dangerous symptoms, weight gain (due to defense eating), deterioration of glycemic control (due to reduced adherence to therapy and therapeutic goals because of fear of new hypoglycemic episodes), increased cardiovascular risk and increased risk for microvascular complications[38-41]. Insulin therapy is associated with a high risk of hypoglycemia[29-31]. In contrast, incretin therapy is associated with a low risk of hypoglycemia[30,31,39-47]. This is because the islet effect of GLP-1 is glucose dependent[7,9] and the glucagon counter-regulation to hypoglycemia is preserved or augmented[48-50]. Therefore, incretin therapy has the potential to prevent the hypoglycemia induced by insulin when the two treatments are used in combination.

Body weight

Since increased body weight is associated with long-term negative effects, prevention of weight gain or weight reduction is of importance for glucose-lowering therapies. Body weight is increased by insulin therapy[51]. This is due to the anabolic action of insulin but may also be due to the self-defense eating associated with hypoglycemic events. Incretin therapy, on the other hand, prevents weight gain since its lowering of glycemia is not associated with increased risk of hypoglycemia and therefore the therapy avoid the self-defense eating[52]. GLP-1 receptor agonists also induce satiety through effects on the satiety center in the hypothalamus, thereby inducing weight reduction[13]. Therefore, the combination of incretin therapy with insulin has a great advantage of preventing the weight gain induced by insulin.

Disease modifying effects

Type 2 diabetes is a progressive disease with is mainly due to a continuous decline in beta cell function[53]. It has been discussed whether insulin therapy and incretin therapy may have complementary disease modifying effects[5]. The rationale for this suggestion is that insulin has been suggested to improve beta cell function through its normalization of fasting glucose, thereby preventing glucotoxicity and may also result in "beta cell rest"[54]. On the other hand, GLP-1 based therapies may improve beta cell function so much that beta cell function will also be improved over a long-term perspective, particularly in association with inhibited beta cell apoptosis[7,9].

ADVANTAGES OF COMBINING INSULIN WITH INCRETIN THERAPY

The complementary actions of insulin and incretin therapy, as discussed above, may result in potential advantages that may be observed by using this combination as a glucose-lowering strategy when treating people with type 2 diabetes. The main advantages are: (1) the combined reduction of fasting and postprandial glycemia which will lower HbA1c; (2) the lower risk of hypoglycemia which is due to the protection against hypoglycemia with incretin therapy in association with the often observed reduction in insulin dose when using this combination; (3) the lower risk for weight gain, which again is due to the protection against weight gain by incretin therapy in association with reduced weight gain through reduction in the insulin dose; and (4) the potential long-term disease modifying prospect of the combination.

CLINICAL STUDIES OF ADDING GLP-1 RECEPTOR AGONISTS TO INSULIN

Exenatide

The first proper clinical trial exploring the combination of incretin therapy with insulin was a study in 259 patients with type 2 diabetes who were treated with insulin glargine (± metformin and/or pioglitazone) with insufficient glycemic control (HbA1c 7.5%-10.5%; mean 8.4%). Patients were randomized to receive additional therapy with exenatide BID (n = 138) or placebo (n = 123) and the dose of insulin glargine was titrated to achieve a fasting glucose level less than 5.6 mmol/L[55]. After the study period of 30 wk, HbA1c was reduced by 1.7% in the group treated with exenatide BID as an add-on compared to 1.0% by placebo ($P < 0.001$). The daily insulin glargine dose had increased by 20 U (95%CI: 16-24) in the placebo group and by 13 U (95%CI: 9-17) in the exenatide BID-group ($P = 0.030$) (baseline insulin glargine dose was 48 U). Postprandial glucose was reduced in the exenatide BID-treated group (by 2.0 mmol/L, 95%CI: 1.5-2.5 mmol/L) but not changed in the placebo group ($P < 0.001$), whereas changes in fasting glucose did not differ between the two groups. Body weight was reduced (by 1.8 kg) in the exenatide BID group but increased (by 1.0 kg) in the placebo-treated group (baseline 94 kg). Furthermore, the number of hypoglycemic events did not differ significantly between the groups inspite of the difference in HbA1c (1.4 episodes per patient year in the exenatide BID-treated group *vs* 1.2 episodes per patient

Table 1 Published clinical trials with glucagon-like peptide-1 receptor agonists added to ongoing insulin therapy

		Exenatide BID		Lixisenatide	
Ref		56	58	59	60
Number of patients		259	495	446	311
Duration (wk)		30	24	24	24
HbA1c	Baseline	8.3 ± 0.9	8.4 ± 0.9	7.6 ± 0.5	8.5 ± 0.7
	Change	-1.7 (-1.9, -1.6)	-0.7 ± 0.1	-0.7 ± 0.1	-0.8 ± 0.2
	Baseline comparator	8.5 ± 1.0	8.4 ± 0.8	7.6 ± 0.5	8.5 ± 0.8
	Change comparator	-1.0 (-1.2, -0.9)	-0.4 ± 0.1	-0.4 ± 0.1	+0.1 ± 0.2
FPG (mmol/L)	Baseline	7.9 ± 2.1	8.1 ± 2.8	6.6 ± 1.7	7.8 ± 2.2
	Change	-1.6 (-1.9, -1.3)	-0.6 ± 0.2	-0.3 ± 0.2	-0.4 ± 0.3
	Baseline comparator	8.3 ± 2.3	8.0 ± 2.7	6.7 ± 2.0	7.7 ± 2.3
	Change comparator	-1.5 (-1.8, -1.2)	-0.6 ± 0.3	-0.5 ± 0.2	0.3 ± 0.3
Hypoglycemia	GLP-1RA	1.4[1]	206[2]	28[3]	42[3]; 33[4]
	Comparator	1.2[1]	522[2]	22[3]	24[3]; 28[4]
Body weight	Baseline	95 ± 20	89 ± 21	88 ± 22	66 ± 13
	Change	-1.8 (-2.4, -1.1)	-1.8 ± 0.2	0.3 ± 0.3	-0.4
	Baseline comparator	93 ± 21	88 ± 20	87 ± 21	66 ± 12
	Change comparator	1.0 (0.2, 1.7)	-0.5 ± 0.3	1.2 ± 0.3	+0.1

Insulin glargine was used in all studies. Occurrence of hypoglycemia was reported as number of episodes per patient year[1], number of events[2] or as percentage of patients with at least one hypoglycemic episode[3]. One study also reported percentage of patients not on sulfonylurea who experienced at least one hypoglycemic episode[4]. Variation in baseline is SD, variation in effect is SE. Variation within parenthesis is the 95%CI. FPG: Fasting glucose; GLP-1: Glucagon-like peptide-1; HbA1c: Hemoglobin A1c.

year in the placebo group) (Table 1).

In another study, a direct comparison was performed between adding exenatide BID *vs* short-acting prandial insulin lispro to ongoing insulin glargine (+ metformin) in patients who were inadequately controlled on insulin glargine + metformin. The study used an initial 12 wk titration phase with insulin glargine [fasting glucose (FPG) glucose target < 5.6 mmol/L]. Patients who failed to reduce HbA1c below 7% during this titration period (mean 8.3%) were randomized to receive additional exenatide BID (n = 316) or insulin lispro (n = 321). The results showed that after 30 wk, HbA1c had been reduced by 1.1% ± 0.1% in both groups (not significantly different). Fasting glucose was reduced by 0.5 ± 0.2 mmol/L in the exenatide group *vs* 0.2 ± 0.2 mmol/L in the insulin lispor group (P = 0.002) and whereas postprandial glucose was similarly reduced after breakfast and evening meals, it was more pronouncedly reduced by lispro at lunch (when exenatide was not given; P < 0.001). Body weight was reduced in the exenatide BID group (by 2.4 ± 0.2 kg) and increased in the insulin lispro group (by 2.1 ± 0.2 kg). The number of hypoglycemic events was lower in the exenatide group (n = 206) than in the insulin lispro group (n = 522)[56].

Lixisenatide

The GLP-1 receptor agonist lixisenatide has been examined as an add-on to basal insulin in three studies. In the first study, patients treated with basal insulin with inadequate glycemic control (HbA1c 7%-10%, mean 7.6%) were randomized to addition of lixisenatide (n = 328) or placebo (n = 167) without any insulin titration[57]. The used basal insulins in the study were insulin glargine (50%), insulin detemir (47%), neutral protamine Hagedorn (NPH) insulin (7%) or premix insulin (2%) and 80% of the patients were additionally treated with metformin. After the study period of 24 wk, HbA1c was reduced by 0.7% by lixisenatide and by 0.4% by placebo (P < 0.001). Fasting glucose was reduced in both groups but with no significant difference. In contrast, postprandial glucose was more pronouncedly reduced in the lixisenatide group (by 5.5 ± 0.5 mmol/L) than in the placebo group (by 1.7 ± 0.5 mmol/L, P < 0.001). Body weight (from baseline of 88 kg) was reduced by 1.8 kg by lixisenatide and 0.5 kg by placebo (P < 0.001). The daily insulin dose (mean 56 U at baseline) had been reduced by 5 U in the lixisenatide group and by 2 U in the placebo group. Twenty-eight percent of patients in the lixisenatide group reported hypoglycemia *vs* 22% in the placebo group.

In the second study on add-on with lixisenatide to basal insulin, lixisenatide was added to insulin glargine in patients who initially failed to control glycemia with oral agents (HbA1c 7%-10%, mean HbA1c 8.6%)[58]. After an initial titration phase of insulin glargine alone for 12 wk targeting a fasting glucose of 4.4-5.6 mmol/L (mean HbA1c was reduced to 7.6%), patients were randomized to lixisenatide (n = 223) or placebo (n = 223) together with ongoing insulin therapy (+ metformin) for 24 wk. It was found that mean HbA1c was further reduced to 7.0% in the lixisenatide group *vs* to 7.3% in the placebo group (P < 0.001). Fasting glucose was similarly reduced in both groups, whereas postprandial glucose was reduced more in the lixisenatide group (by 3.4 ± 0.5 mmol/L) than in the placebo group (0.1 ± 0.5 mmol/L; P < 0.001). Body weight was increased by 1.2 kg in the placebo group and by 0.3 kg in the lixisenatide group (baseline 86 kg) (P = 0.0012). Confirmed hypoglycemia was reported in 0.80 episodes per patient year in the lixisenatide group *vs* 0.44 in the placebo group.

Finally, the effect of adding lixisenatide to ongoing insulin therapy has also been examined in Asian patients with inadequate glycemic control on basal insulin [with (70%) or without sulfonylurea therapy][59]. Of the patients, 60% were treated with insulin glargine, 27% with insulin detemir and 13% with NPH insulin with a mean daily insulin dose of 25 U. The patients were randomized to addition of lixisenatide (n = 154) or placebo (n = 157) together with ongoing therapy with basal insulin ± sulfonylurea. After the 24 wk study period, HbA1c was reduced by 0.8% in the lixisenatide group *vs* increased by 0.1% in the placebo group (P < 0.001). There was a reduction in fasting glucose in the lixisenatide group compared to the placebo group (P = 0.0187) and postprandial glucose was reduced by 8 mmol/L in the lixisenatide group but not changed in the placebo group (P < 0.001).

Table 2 Published clinical trials with dipeptidyl peptidase-4 inhibitors combined with basal ± prandial insulin

		Vildagliptin		Sitagliptin		Alogliptin	Saxagliptin	Linagliptin
Ref		62	63	64	65	67	69	70
Number of patients		296	449	641	124	390	455	1261
Study duration (wk)		24	24	24	24	26	52	24
Comparator		Stable insulin	Stable insulin	Stable insulin	Increasing insulin	Stable insulin	Stable insulin	Stable insulin
HbA1c (%)	Baseline	8.4 ± 1.0	8.8 ± 1.0	8.7 ± 0.9	9.2 ± 1.0	9.3 ± 1.1	8.7 ± 0.9	8.3 ± 0.1
	Change	-0.5 ± 0.1	-0.8 ± 0.1	-0.6 (-0.7, -0.5)	-0.6 (-0.9, -0.3)	-0.7	-0.8 ± 0.1	-0.6 ± 0.1
	Baseline placebo	8.4 ± 1.1	8.8 ± 1.0	8.6 ± 0.9	9.2 ± 1.1	9.3 ± 1.1	8.6 ± 0.9	8.3 ± 0.1
	Change placebo	-0.2 ± 0.1	-0.1 ± 0.1	0 (-0.1, 0.1)	-0.2 (-0.5, 0.3)	-0.1	-0.4 ± 0.1	-0.1 ± 0.1
FPG (mmol/L)	Baseline	9.3 ± 3.1	9.6 ± 2.6	9.8 ± 2.9	9.0 ± 3.3	10.3 ± 3.9	NR	8.2 ± 2.6
	Change	-0.8 ± 0.3	-0.8	-1.0 (-1.4, -0.7)	-1.0 (-2.7, -0.2)	-0.6 ± 0.3		-0.2 ± 0.2
	Baseline placebo	8.7 ± 3.1	9.1 ± 2.5	9.9 ± 3.3	8.4 ± 2.8	10.9 ± 4.3	NR	8.4 ± 2.6
	Change placebo	-0.2 ± 0.4	-0.2	-0.2 (-0.6, 0.2)	-1.3 (-1.8, -0.5)	0.3 ± 0.3		-0.3 ± 0.2
Hypoglycemia		113[1]	8.4[2]	16[2]	7[2]	20[2]	23[2]	23[2]
Hypoglycemia placebo		185[1]	7.2[2]	8[2]	14[2]	40[2]	27[2]	22[2]
Body weight (kg)	Baseline	95 ± 2	78 ± 16	87 ± 19	69 ± 12	87 ± 19	88 ± 18	BMI (31 ± 5)
	Change	1.3 ± 0.3	0.1	-0.1 (-0.2, 0.4)	-0.7 (-1.4, -0.1)	0.6 ± 0.2	0.8	-0.2 ± 0.1
	Baseline placebo	95 ± 2	79 ± 17	87 ± 18	66 ± 10	91 ± 21	86 ± 16	BMI (31 ± 5)
	Change placebo	0.6 ± 0.3	-0.4	-0.1 (-0.3, 0.4)	1.1 (0.2, 1.8)	0.6 ± 0.2	0.5	0.1 ± 0.1

In the studies long and medium acting insulin and premixed insulins were used. Occurrence of hypoglycemia was reported as number of events[1] or as percentage of patients with at least one hypoglycemic episode[2]. Variation in baseline is SD, variation in effect is SE. Variation within parenthesis is the 95%CI. FPG: Fasting glucose; BMI: Body mass index (kg/m^2); HbA1c: Hemoglobin A1c.

Symptomatic hypoglycemia was more frequent with lixisenatide (42.9%) *vs* placebo (23.6%). In contrast, in patients not treated with sulfonylurea, hypoglycemia was similar between groups (32.6% *vs* 28.3%, respectively). Change in body weight was not significantly different between the groups whereas the daily insulin dose was reduced by 1.4 U in lixisenatide group *vs* by 0.1 U in the placebo group ($P = 0.0019$).

Albiglutide

A study compared the effects of the once weekly GLP-1 receptor agonist albiglutide ($n = 285$) *vs* insulin lispro ($n = 281$) to ongoing insulin glargine therapy (+ oral agents, no sulfonylurea) in patients with type 2 diabetes with inadequate glycemic control (mean HbA1c 8.5%)[60]. There was a titration algorithm for insulin glargine to achieve fasting glucose of 4.4-7.2 mmol/L. After the 26 wk study period, HbA1c was similarly reduced by albiglutide (0.8% ± 0.1%) and by insulin lispro (0.7% ± 0.2%). Fasting glucose was reduced in both groups with no significant difference. Body weight (baseline 92 kg) was reduced 0.7 ± 0.2 kg by albiglutide and increased by 0.8 ± 0.2 kg by insulin lispro ($P < 0.001$). Mean insulin glargine dose did not change during the study. Thirty-two percent of patients on albiglutide experienced hypoglycemia *vs* 50% with insulin lispro.

CLINICAL STUDIES OF ADDING DPP-4 INHIBITORS TO INSULIN

Vildagliptin

The first study examining a DPP-4 inhibitor in combination with insulin added vildagliptin (*vs* placebo) to insulin treated patients with insufficient glycemic control (HbA1c 7.5%-11%, mean HbA1c 8.4%; $n = 296$)[61]. Patients were treated with basal and prandial insulin (mean 2.8 injections per day, mean daily insulin dose 82 U). After the 24 wk study period, HbA1c was reduced by 0.5% in the vildagliptin group *vs* 0.2% in the placebo group (baseline 8.4%) ($P = 0.01$). During the course of the study, there were 113 hypoglycemic events in the vildagliptin group compared to 185 in the placebo group and whereas there were 6 episodes of severe hypoglycemia in the placebo group, no severe hypoglycemic episode was seen in the vildagliptin group. The mean daily insulin dose was reduced by 1.9 U in the vildaglitpin *vs* increased by 2.4 U in the placebo group. Change in body weight did not differ between the groups (Table 2).

Another study examined the addition of vildagliptin to ongoing insulin (+ metformin) therapy in 449 patients over 24 wk[62]. The patients were treated with long-acting insulin (22%), intermediate acting insulin (17%) and premixed insulin (60%), with a mean daily insulin dose of 40 U. They had insufficient glycemic control (HbA1c 7.5%-11%; mean HbA1c 8.8%). It was found that HbA1c was reduced by vildagliptin by 0.8% and by placebo by 0.1% ($P < 0.001$). Fasting glucose was reduced in the vildagliptin group but not in the placebo group ($P = 0.050$). Hypoglycemia was reported in 8.4% of patients in the vildagliptin group and by 7.2% in the placebo group. The daily insulin dose was 41 U at baseline and slightly reduced in both groups with no difference. There was no change in body weight in any of the groups.

Sitagliptin

The first study examining the combination of sitagliptin with insulin therapy added the DPP-4 inhibitor *vs* placebo to ongoing insulin (+ metformin) treatment over 24 wk in 641 patients with poorly controlled type 2 diabetes (HbA1c 7.5%-11%, mean HbA1c 8.6%)[63]. Seventy-four percent of the patients were treated with long-acting

or intermediate-acting insulin and 26% were treated with premixed insulin. After the 24 wk study period, HbA1c was reduced by 0.6% by sitagliptin *vs* no change by placebo ($P < 0.001$). Fasting glucose was reduced in the sitagliptin group but not in the placebo group ($P < 0.001$). Similarly, postprandial glucose was reduced in the sitagliptin group (by -1.7 mmol/L, 95%CI: -2.2, -1.2) but not changed in the placebo group (0.3 mmol/L, 95%CI: -0.2-0.7) ($P < 0.001$). Hypoglycemia was observed in 16% of the patients on sitagliptin *vs* 8% of patients on placebo. Insulin dose was reduced by 0.1 U in the sitagliptin and by 1.6 U in the placebo group (baseline 44 U for long acting insulin and 67-74 U with premixed insulin). Body weight was reduced by 0.1 kg in both groups.

Another study compared adding sitagliptin to insulin therapy *vs* increasing the insulin dose in 140 patients on insulin therapy (+ oral agents) who had inadequate glycemic control (baseline HbA1c 7.5%-11%, mean HbA1c 9.2%). Patients were treated with insulin glargine alone (48%), insulin glargine together with rapid acting insulin (23%) or NPH insulin in combination with regular insulin (29%); mean daily insulin dose was 37 U. It was found that over the 24 wk study period, sitagliptin (mean insulin dose reduced by 2 U) reduced HbA1c by 0.6%, whereas increasing the insulin dose (by 10 U) reduced HbA1c by 0.2% ($P < 0.005$)[64]. Fasting glucose was reduced by approximately 1 mmol/L in both groups with no significant difference. Hypoglycemia occurred in 7 events per patient year in the sitagliptin group *vs* 14.3 events per patient year in the insulin group. Body weight was reduced by 0.7 kg in the sitagliptin group *vs* increased by 1.1 kg in the insulin group ($P < 0.05$).

A third study examined the add-on of sitagliptin ($n = 236$) *vs* placebo ($n = 232$) to patients who were treated with insulin (long-acting, intermediate-acting or premixed insulin) in combination with metformin over 6 mo. It was found that with the addition of sitagliptin, HbA1c was reduced by 0.8% (baseline 8.5%) *vs* no change in HbA1c after addition of placebo ($P < 0.001$). Relative to the placebo group, fasting glucose was reduced by 1.0 mmol/L and postprandial glucose by 2.0 mmol/L. Hypoglycemia was observed in 18% of patients in the sitagliptin group *vs* 8% in the placebo group[65].

Alogliptin

Alogliptin (two doses) or placebo was added to ongoing insulin therapy alone (40%) or with metformin in 390 patients with inadequate glycemic control (HbA1c ≥ 8.0%; baseline HbA1c 9.3%)[66]. The insulin treatment that was used was premixed insulin or insulin combinations (64%), as well as long-acting basal insulin alone (34%) or short-acting insulin alone (2%); mean daily insulin dose was 57 U. During the course of the 26 wk study, daily insulin dose was kept constant. Alogliptin reduced HbA1c by 0.6% (12.5 mg daily; $n = 131$) and 0.7% (25 mg daily; $n = 129$) *vs* a reduction by 0.1% in the placebo group ($n = 130$) ($P < 0.001$). Fasting glucose was reduced by aloglitin in the 25 mg group (by -0.6 ± 0.3 mmol/L *vs* the placebo group (0.3 ± 0.3 mmol/L; $P = 0.030$) but not changed in the 12.5 mg group. The number of patients reporting hypoglycemia was lower in the two alogliptin groups (21% and 20%, respectively) than in the placebo group (40%; $P < 0.001$). There was no difference in hypoglycemia events (24%-27% of patients reported hypoglycemic episodes in the three groups). Body weight increased by 0.6 kg (baseline 88 kg) in all groups.

Saxagliptin

Saxagliptin or placebo was added to ongoing insulin therapy (basal insulin or premixed insulin ± metformin) in 455 patients with inadequate glycemic control (HbA1c 7.5-11). During the course of the 24 wk study, daily insulin dose was kept constant[67]. Placebo-adjusted reduction in HbA1c by saxagliptin was 0.4% ($P < 0.001$). There was no difference in hypoglycemia events (18% with saxagliptin, 20% with placebo). Body weight was increased by 0.4 kg in the saxagliptin group and by 0.2 kg in the placebo group. An extension phase of this study showed sustained effects over 52 wk[68].

Linagliptin

Linagliptin or placebo was added to ongoing basal insulin therapy (± metformin and/or pioglitazone) in 1261 patients with inadequate glycemic control (HbA1c 7-10). During the study, daily insulin dose was kept constant during the first 24 wk but could thereafter be titrated according to fasting glucose[69]. After 24 wk, HbA1c was reduced by 0.6% (baseline 8.3%) by linagliptin and by 0.1% by placebo ($P < 0.001$). Placebo-adjusted reduction in fasting glucose with linagliptin was 0.6 mmol/L (95%CI: -0.9-0.4). During the following 28 wk, insulin dose was increased by 2.6 U in the linagliptin group and by 4.2 U in the placebo group but with no further change in HbA1c. There was no difference in hypoglycemia events (23% with linagliptin, 22% with placebo after 24 wk). Body weight was reduced by 0.3 kg in the linagliptin group and by 0.04 kg in the placebo group.

COMPARING CONTROLLED TRIALS COMBINING ADDING INCRETIN THERAPY TO INSULIN

As outlined above, the reduction in HbA1c, fasting and postprandial glucose, the lower risk of hypoglycemia, the prevention of weight gain and the potential disease modification are the main advantages of combining incretin therapy with insulin. Except for any direct evidence of a disease modifying effect of the combination, the controlled trials summarized above include information on these aspects and therefore it is of interest to compare their results in this regard (Tables 1 and 2).

HbA1c

The mean reduction in HbA1c in the controlled clinical studies adding incretin therapy to stable dose for 6 mo

was -0.8% ± 0.1% compared to -0.3% ± 0.1% when placebo was added ($P < 0.001$; Tables 1 and 2). There does not seem to be a difference between the two different strategies of incretin therapy since the placebo-adjusted reduction in HbA1c was -0.6% ± 0.2% for GLP-1 receptor agonists ($n = 4$ studies) *vs* -0.5% ± 0.1% for DPP-4 inhibitors ($n = 6$ studies).

Fasting glucose

Fasting glucose is also reduced by adding incretin therapy to stable dose of insulin. It was found to be reduced by -0.7 ± 0.1 mmol/L by the incretin therapy *vs* by -0.3 ± 0.1 mmol/L in the placebo groups ($P = 0.027$; Tables 1 and 2). There does not seem to be a difference between the two different strategies of incretin therapy since fasting glucose was reduced by 0.2 ± 0.2 mmol/L by GLP-1 receptor agonists ($n = 4$ studies) *vs* by -0.6 ± 0.2 mmol/L by DPP-4 inhibitors ($n = 5$ studies).

Postprandial glucose

A few studies also examined postprandial glucose after adding incretin therapy to a stable dose of insulin. They showed that postprandial glucose was markedly reduced when adding GLP-1 receptor agonists exenatide BID[55] and lixisenatide[58], whereas after adding the DPP-4 inhibitor sitagliptin, postprandial glucose was more modestly reduced[63].

Hypoglycemia

In the studies where incretin therapy has been added to insulin compared to ongoing insulin, the occurrence of hypoglycemia was not different between the incretin treatment and placebo in most studies (Tables 1 and 2). Since in most of these studies HbA1c is lower after addition of incretin therapy compared to placebo, an increased risk of hypoglycemia would be expected after incretin therapy. Since the opposite was the case, a conclusion is that incretin therapy will reduce the risk of hypoglycemia. This conclusion is also evident in the studies in which incretin therapy as an add-on to basal insulin was compared with the active comparator of either adding short-acting insulin[56,60] or increasing the insulin dose[64]. A reason for the low risk of hypoglycemia when adding incretin therapy to insulin therapy could be the reduced dose of insulin which often accompanies the combination. It may, however, also be caused be a sustainment of the glucagon counterregulation to hypoglycemia, as was recently demonstrated for the DPP-4 inhibitor vildagliptin when added to insulin; the sustained glucagon counterregulation assures a sufficient hepatic glucose response to prevent hypoglycemia[50].

Weight gain

Body weight was significantly reduced by -0.9 ± 0.5 kg by adding GLP-1 receptor agonists to ongoing insulin therapy compared to 0.4 ± 0.4 kg in the placebo groups, corresponding to a placebo-adjusted reduction by -1.4 ± 0.5 kg (Table 1). In contrast, DPP-4 inhibitors are weight neutral when added to insulin with a placebo-adjusted change in body weight of -0.2 ± 0.1 kg (Table 2).

OTHER STUDIES COMBINING INCRETIN THERAPY WITH INSULIN

Adding insulin to a GLP-1 receptor agonist

One study has examined the addition of basal insulin to patients who are treated with a GLP-1 receptor agonist with insufficient glycemic control. The study initially examined addition of liraglutide to patients failing glycemic control on metformin (± sulfonylurea; sulfonylurea was removed at start of study) ($n = 988$)[70]. After 12 wk, patients who were still uncontrolled (HbA1c > 7%) were randomized to continue metformin plus liraglutide or addition of insulin detemir to titrate fasting glucose to 4-6 mmol/L. After another 26 wk, HbA1c had been reduced by 0.5% by the combination of insulin detemir plus liraglutide, whereas those on liraglutide alone (all with metformin) had no further change in HbA1c ($P < 0.001$). FPG decreased more in the liraglutide + insulin group than in the liraglutide control group ($P < 0.001$). Hypoglycemia rates were 9.2% in the group given insulin detemir and liraglutide *vs* 1.3% with liraglutide alone. Body weight (baseline 96 kg) decreased by 3.5 kg by liraglutide during the initial period and then by 0.16 kg with insulin detemir and liraglutide *vs* by 0.95 kg with liraglutide without insulin detemir ($P = 0.03$).

Initial combination of incretin therapy with insulin

Liraglutide has been examined in a fixed ratio combination with insulin degludec in a randomized study in subjects with type 2 diabetes[71]. It was a large trial in which patients treated with metformin ± pioglitazone and inadequate glycemic control (baseline HbA1c 8.3%) were randomized to the addition of insulin degludec ($n = 414$), liraglutide ($n = 415$) or the combination of insulin degludec and liraglutide ($n = 834$). After 26 wk of treatment, HbA1c had been reduced by 1.4% with insulin degludec alone, 1.3% with liraglutide alone and 1.9% with insulin degludec in combination with liraglutide. Body weight had increased by 2.2 kg with insulin degludec alone, was reduced by 2.4 kg by liraglutide and was neutral with the combination. Cumulative episodes of hypoglycemia were 1.3 per patient in the insulin degludec group and reduced to 0.9 per patient in the combination group (0.1 in the liraglutide alone group).

Another study randomized 217 patients who had insufficient glycemic control on metformin ± sulfonylurea to receiving sitagliptin plus sulfonylurea or sitagliptin plus insulin detemir (all on metformin). After the 26 wk study period, sitagliptin had reduced HbA1c by 0.9% (mean baseline HbA1c 8.5%), whereas sitagliptin plus insulin detemir had decreased HbA1c by 1.4%. Hypoglycemia was reported in 1.3% of patients in the insulin detemir plus sitagliptin group and 1.7% in the sitagliptin alone group. Body weight decreased in both arms with a mean decrease of -1.7 kg in the sitagliptin control group *vs* -0.8 kg with sitagliptin plus insulin detemir group[72].

Uncontrolled studies combining incretin therapy with insulin

There are also a few uncontrolled studies of combining insulin therapy and incretin therapy in patients with type 2 diabetes which arrive at similar conclusions as the previously summarized controlled trials. One retrospective report showed that addition of exenatide BID to 188 insulin-treated patients resulted in a reduction in HbA1c by 0.66% (baseline 8.1%) after 6 mo with a persistent effect throughout two years; mean insulin dose could be reduced by 15% and only 4% of patients experienced hypoglycemia[73]. Furthermore, a study in obese patients with type 2 diabetes added exenatide BID (n = 21) or liraglutide (n = 40) to ongoing insulin therapy and showed a reduction in HbA1c in these patients by 1.0% (baseline 8.9%) after 7 mo. At the same time, the daily insulin dose was reduced from 91 U to 52 U and only a few hypoglycemia episodes were reported[74]. Moreover, a study in severely insulin resistant obese subjects treated with insulin U-500 (mean daily dose 192 U) received liraglutide for twelve weeks which reduced HbA1c by 1.4% (mean baseline HbA1c 8.5%) and at the same time the insulin dose was reduced by 28%. There were no reports of hypoglycemia and body weight was reduced by 5 kg (baseline body weight 136 kg)[75].

SAFETY OF THE COMBINATION OF INSULIN AND INCRETIN THERAPY

Incretin therapy has been shown to be safe with high tolerability and the only consistent adverse event is nausea and vomiting during the initial treatment period with GLP-1 receptor agonists[17-20,76]. Local injection site reactions (nodules and/or erythema) sometimes occur in association with treatment with GLP-1 receptor agonists, although such reactions are rare and commonly transient. Antibodies may be formed against GLP-1 receptor agonists; more commonly with exendin-4-based agonists (exenatide, lixisenatide) than after GLP-1-based agonists. In contrast, adverse events are rare during treatment with DPP-4 inhibitors, as evident from pooled analysis of clinical trials[77,78]. Recently, there has been a discussion about whether there is an increased risk of acute pancreatitis in incretin therapy. However, pooled or meta-analysis analyses have not demonstrated any increased risk when compared to placebo or other comparators[76-79]. Nevertheless, it is important to follow patients on GLP-1 receptor agonists in this regard and in patients with a history of acute pancreatitis, incretin therapy should not be given. Rodent data also suggest that GLP-1 receptor agonists may be associated with medullary thyroid carcinoma[80]. This has not, however, been observed in other animal species or humans, possibly because C-cells in humans have a lower expression of GLP-1 receptors than rodent C-cells[81].

Incretin therapy has also been discussed in relationship to cardiovascular safety and meta-analyses have shown that there is no detrimental effect of GLP-1 receptor agonists[82] or DPP-4 inhibitors[83]. Furthermore, several cardiovascular safety trials with incretin therapy are at present ongoing and two such recently published studies showed no increased risk for cardiovascular disease with saxagliptin[84] or alogliptin[85].

Also, insulin therapy is safe with the only concern being the increased risk of hypoglycemia and weight gain, expected adverse events through the glucose-lowering actions of the therapy. By combining incretin therapy and insulin, there is no additional concern for safety or tolerability, as evident from the studies reported in this review[55-69]. Hence, the number of adverse events is not higher in the incretin therapy + insulin treatment groups than in placebo groups in placebo-controlled clinical trials on GLP-1 receptor agonists or DPP-4 inhibitors as an add-on to insulin therapy, except the nausea and vomiting for the GLP-1 receptor agonists. This also includes recently discussed potential adverse events such as acute pancreatitis.

Some practical aspects need to be taken into account for incretin therapy. This includes the dose reduction of sitagliptin, vildagliptin, saxagliptin and alogliptin in patients with renal impairment due to their renal excretion. Furthermore, exenatide and liraglutide should be cautiously used in patients with renal impairment due to insufficient experience in this patient group. Furthermore, in patients with hepatic impairment, vildagliptin is not recommended. As for all new treatment combinations, however, the combination of incretin therapy with insulin also needs careful follow-up for examining potential adverse events which have not yet been observed.

SUMMARY AND CLINICAL POSITIONING OF INCRETIN PLUS INSULIN COMBINATION

The combination of insulin therapy with incretin therapy is attractive due to experience that this combination improves glycemia with a low risk of increasing risk for hypoglycemia and low risk of weight gain. The combination is therefore of particular value in patients on insulin therapy in whom HbA1c is not sufficiently reduced. In some patients, insufficient improvement of glycemia may be caused by clinical inertia with reluctance to increase the insulin dose due to fear of hypoglycemia or weight gain. Addition of incretin therapy with its lower risk of hypoglycemia and low risk of weight gain may therefore prevent the clinical inertia in these patients.

Incretin addition is also of value in patients who have insufficient reduction in HbA1c by intensified basal insulin therapy due to persistent high postprandial glycemia or frequent hypoglycemia. Incretin therapy offers advantages over addition of prandial insulin in these patients. Of particular importance is the prevention of hypoglycemia, since hypoglycemia is associated with both short-term and long-term negative impact, not least on cardiovascular outcomes. The combination of incretin therapy with insulin may therefore provide advantages both in the short-term and by reducing long-term complications

to the disease.

A main indication for the combination of incretin therapy and insulin is thus in patients who are treated with basal insulin (± metformin) in whom there is insufficient glycemic control and/or an unacceptable high rate of hypoglycemia and/or unacceptable weight gain. In patients with HbA1c levels which are not very high (< 7.5%), it is advisable to reduce the basal insulin dose when starting incretin therapy. The combination of incretin therapy with insulin is also an important treatment strategy in patients who are treated with metformin and incretin therapy in combination and in whom the glycemic control is insufficient, *i.e.*, to add basal insulin therapy to incretin therapy (+ metformin). The combination with incretin therapy and insulin may thus be introduced in either way, starting with incretin therapy or starting with insulin. It is also a possibility to start immediately with initial combination with incretin therapy and insulin in patients who are treated with metformin and who are in insufficient metabolic control. Such an early introduction of the combination may be a solution to the unmet need to start aggressive therapy early on during the disease development to achieve long-term control. Further studies are required to examine the long-term effects of this initial combination. One important set of trials would be studies comparing this treatment strategy with other three-drug combinations. This would be of interest to further analyze the potential for the combination of incretin plus insulin therapy (+ metformin). What would also be of value would be to compare different incretin therapies (different GLP-1 receptor agonists and different DPP-4 inhibitors) to elucidate potential differences in effects of the different compounds when combined with insulin. More mechanistic studies would also be of value, for example to examine the relationship between insulin therapy and incretin hormones for the regulation of hepatic glucose output, glucose utilization and islet function and, furthermore, to study impact of the combination therapy on gastric emptying and satiety. Moreover, it would also be of great value to analyze the cardiovascular outcome of this three-drug combination. This would be possible in sub-group analysis on the cardiovascular outcome trials of incretin treatment in which patients on insulin therapy have also been enrolled. Finally, studies directly aiming at examining the potential disease modifying effect of the combination of incretin therapy and insulin are important; these studies need to have a long duration and include mechanistic studies on islet function.

The combination of incretin therapy with insulin (± metformin) is thus a promising glucose-lowering strategy in type 2 diabetes, allowing a more intensified treatment at an earlier stage of the disease with a lower risk for hypoglycemia and weight gain when compared to other intensifying therapies.

ACKNOWLEDGMENTS

The author has consulted for AstraZeneca, BMS, Boehringer Ingelheim, SK, Merck, Novartis, Novo Nordisk, Sanofi-Aventis and Takeda, all of which produce GLP-1 receptor agonists or DPP-4 inhibitors.

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P- Reviewers: Chang ST, Faerch K, Liu SH, Rateb M
S- Editor: Cui XM **L- Editor**: Roemmele A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Expression quantitative trait analyses to identify causal genetic variants for type 2 diabetes susceptibility

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Author contributions: Das SK performed the literature review, integrated key scientific concepts, and wrote the paper; Sharma NK performed the literature and data mining and reviewed the manuscript.

Supported by National Institutes of Health (NIH/NIDDK), Nos. R01 DK090111 and DK039311

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Received: November 27, 2013 Revised: February 21, 2014
Accepted: March 13, 2014
Published online: April 15, 2014

Abstract

Type 2 diabetes (T2D) is a common metabolic disorder which is caused by multiple genetic perturbations affecting different biological pathways. Identifying genetic factors modulating the susceptibility of this complex heterogeneous metabolic phenotype in different ethnic and racial groups remains challenging. Despite recent success, the functional role of the T2D susceptibility variants implicated by genome-wide association studies (GWAS) remains largely unknown. Genetic dissection of transcript abundance or expression quantitative trait (eQTL) analysis unravels the genomic architecture of regulatory variants. Availability of eQTL information from tissues relevant for glucose homeostasis in humans opens a new avenue to prioritize GWAS-implicated variants that may be involved in triggering a causal chain of events leading to T2D. In this article, we review the progress made in the field of eQTL research and knowledge gained from those studies in understanding transcription regulatory mechanisms in human subjects. We highlight several novel approaches that can integrate eQTL analysis with multiple layers of biological information to identify ethnic-specific causal variants and gene-environment interactions relevant to T2D pathogenesis. Finally, we discuss how the eQTL analysis mediated search for "missing heritability" may lead us to novel biological and molecular mechanisms involved in susceptibility to T2D.

Key words: Type 2 diabetes; Single nucleotide polymorphisms; Expression quantitative trait locus; Expression regulatory SNPs; Gene-environment interaction; Genome-wide association study

Core tip: Identification of genetic variants that modulate the susceptibility to disease and elucidating their function at the molecular level is a major focus of type 2 diabetes (T2D) research. This article highlights the utility of expression quantitative trait analysis in discovering regulatory variants that increase susceptibility to T2D by modulating the expression of transcripts in tissues important for glucose homeostasis.

Original sources: Das SK, Sharma NK. Expression quantitative trait analyses to identify causal genetic variants for type 2 diabetes susceptibility. *World J Diabetes* 2014; 5(2): 97-114 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/97.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.97

GENETIC DISSECTION OF TYPE 2 DIABETES SUSCEPTIBILITY

Diabetes is one of the most prevalent metabolic disorders, characterized by elevated levels of plasma glucose,

and is responsible for significant mortality and morbidity in human populations worldwide[1]. The latest estimate from the International Diabetes Federation indicates a global prevalence rate of 8.4% in adults and 382 million cases of diabetes in 2013[2]. It is one of the common diseases with a well-accepted genetic contribution[3]. Type 2 diabetes (T2D), a late onset subtype of diabetes, results from a derangement in the complex interplay of multiple physiological processes known to be involved in systemic glucose homeostasis. These processes include peripheral glucose uptake in muscle, secretion of hormones and incretins form pancreas and intestine, secretion of cytokines/adipokines from adipose tissue, hepatic glucose production, and neuro-endocrine regulation by central nervous system[4,5]. However, the relative contribution of these processes to T2D pathogenesis is debated. Based on this knowledge on intertwined and complex physiological processes it can be anticipated that T2D is a heterogeneous conglomeration of phenotypes, caused by multiple genetic perturbations and affecting different biological pathways. Predictably, deciphering the genetic etiology of T2D has remained challenging.

Until the last decade, searching for an association between T2D and sequence variants of selected candidate genes was the mainstay of research for finding genetic susceptibility factors. Based on available technology in those studies, researchers selected candidate genes either from loci detected by genome-wide linkage analyses or based on known physiological functions. In our earlier reviews, we discussed the knowledge gained from such studies in detail[6,7]. Success from those endeavors was very limited. However, this approach has identified genetic variants in the *TCF7L2* gene, to date is the best replicated and strongest (relative risk approximately 1.4) genetic susceptibility factor for T2D[8], but its role is still controversial[9-11].

In the middle of the last decade, a transformative change took place in the field of genetics of complex disease research. Advances in high-throughput genotyping technology, availability of the complete human genome sequence, a dense catalogue of common genetic variants, and a population-specific linkage disequilibrium map of these variants lead to the implementation of genome-wide association studies (GWAS), which interrogate the entire genome to identify common genetic variants (minor allele frequency $\geqslant 0.05$) associated with a disease[12]. GWAS have yielded unprecedented success in identifying well-replicated susceptibility loci for T2D, glucose homeostasis traits, obesity, and related metabolic phenotypes[3,13-15]. Nevertheless, these successes come with significant caveats. Based on the most recent analyses, the 63 T2DM-associated loci discovered so far in Caucasian populations together account only for 5.7% of the liability-scale variance in disease susceptibility, and sibling relative risk (λ_s) attributed jointly by these variants is 1.104[13]. Moreover, few of the T2D loci identified primarily in European- or Asian-derived populations are convincingly replicated in African American, Native American, and Hispanic populations, all of whom have a higher prevalence of T2D than Caucasians[14,16]. These GWAS-identified loci do not appear to explain the well-established roles for adipose, muscle, and liver in diabetes pathogenesis[17], and few of these loci have been linked to a molecular mechanism. Several investigators have attempted to implicate function to T2D-associated loci based on their proximity to a gene, assuming that the associated single nucleotide polymorphisms (SNP) alters the function of a nearby gene[18]. Some have drawn enthusiastic conclusions about the role of these variants exclusively in insulin secretion[19]. However, proof of such an assumption is lacking. Given the small effect on T2D susceptibility and the statistical noise inherent in performing 10^6 or more tests, exclusive reliance on larger T2D GWAS alone is unlikely to identify the source of undefined T2D susceptibility (often referred to as "missing heritability"[20]).

EXPRESSION QUANTITATIVE TRAITS: MOLECULAR ENDOPHENOTYPES

One of the major findings from the T2D GWAS is that most of the trait-associated SNPs are located in intronic, intergenic, or other non-coding regions of the genome[3,21]. Further fine mapping analysis also failed to find any coding or other variants that would provide a molecular biological explanation of the elevated disease risk attributed by these loci.

The abundance of a transcript is a quantitative trait. Studies in human populations showed a wide, heritable variation of transcript levels among individuals, and thus lead to the concept of "expression quantitative trait loci" (eQTL)[22,23]. The heritability of eQTLs has been replicated in multiple human tissue or cell types, with approximately 30% of eQTLs having $h^2 > 0.3$, and an estimated 58%-85% being heritable[24-28]. The abundance of a transcript can be directly modified by polymorphisms in non-coding regulatory elements. Many SNPs are associated with quantitative transcript levels and are considered as expression regulatory SNPs (eSNPs). eSNPs close to the transcription start sites (TSS) of the eQTLs are named "*cis*" or "local" eSNPs , whereas eSNPs located $> \pm 500$ kb from the TSS or on a different chromosome are considered "*trans*" or "distal" eSNPs[22,29]. Similar to a published study[30], here we will refer to eQTLs as the transcripts rather than SNP-transcript pair, and eSNPs as the genetic variants (SNPs) associated with the expression profile of a transcript.

Based on this knowledge, many laboratories (including ours) hypothesized that GWAS-associated non-coding variants are eSNPs and can modulate T2D susceptibility by altering transcript levels (or splicing). This concept is based on the "central dogma" of gene expression and presents a causal model of genetic susceptibility (Figure 1). In this model, transcript abundance is considered as an intermediate phenotype between genetic loci (DNA sequence variants) and subclinical (*e.g.*, insulin resistance) or clinical (*e.g.*, T2D) phenotypes. Since transcript abun-

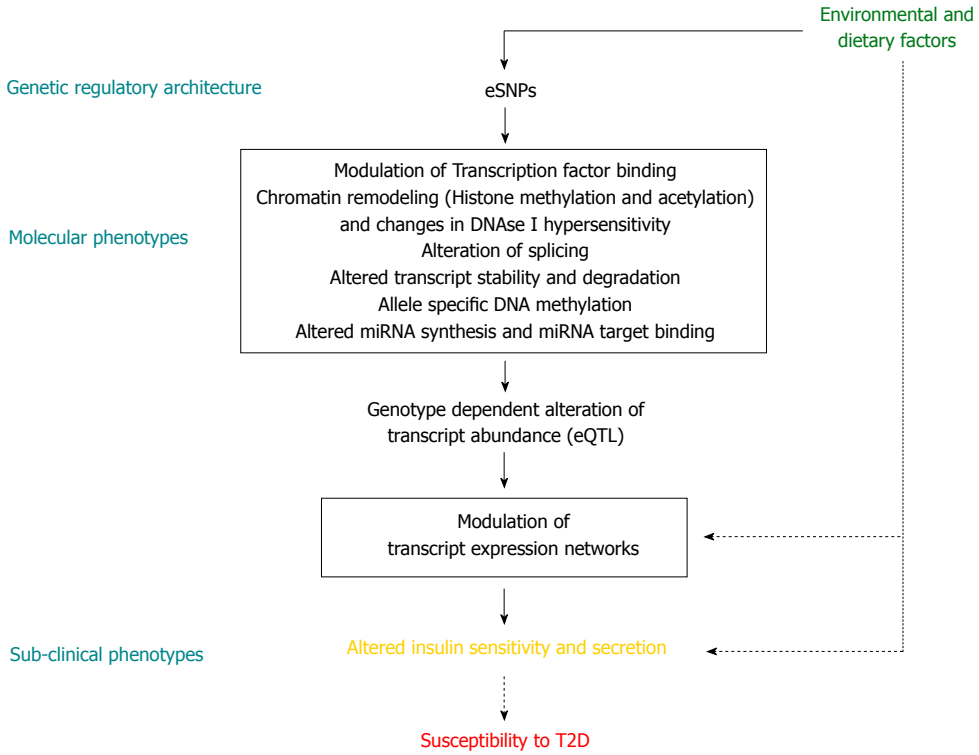

Figure 1 A causal model of genetic susceptibility. Genetic regulatory architecture modulates molecular phenotypes in interaction with environmental factors and alters disease susceptibility. eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

dance is a proximal molecular endophenotype affected by genetic variants, it is likely to be a less heterogeneous phenotype (compared to complex clinical phenotypes like those of T2D), and thus more amenable to genetic mapping methods due to superior statistical power.

EQTL MAPPING

Study designs and analytical frameworks for eQTL mapping are similar to those for mapping any other quantitative traits [*e.g.*, body mass index (BMI), fastin glucose, glycosylated hemoglobin]. However, genetic analysis of human phenotypes including QTLs carries a unique set of problems[29]. In general, eQTL analyses integrate genome-wide expression (in tissues or cells) and genotype data in multiple individuals (related or unrelated). These analyses use linkage- or association-based statistical genetic methods to map regulatory regions and genetic variants that may explain individual variations in transcript expression. Microarray- or RNA-seq[31-33] based methods are used to generate large numbers of quantitative transcript phenotypes. Therefore, the number of statistical tests involved in eQTL mapping studies is significantly higher than in traditional QTL analysis[34]. A detailed discussion on methods used in eQTL analysis is beyond the scope of this article, and we refer our readers to other reviews on this specific subject[29,34-36].

Published eQTL studies have implemented linkage analysis by using 400-2000 microsatellite makers[24,26] to localize regulatory intervals, whereas other studies have genotyped large numbers of common SNPs (> 100000) to discover the eSNPs[25,28,37] associated with eQTLs. With the advancement of genomic technology, we can now simultaneously genotype more than 4.5 million SNPs or can have a whole genome sequence for each individual included in an eQTL study by highly multiplexed "next generation" sequencing methods[38]. These advances pose additional statistical and computational challenges, and will require appropriate correction and adjustment of significance thresholds for the massive number of independent tests performed (and hypotheses tested) to control false discovery. The power to detect eSNPs depends on their effects (average difference in the transcript abundance between genotypes, scaled by the standard deviation of the transcript abundance within genotype classes) and allele frequency[34]. Consequently, detection of eSNPs with a lower effect allele frequency and a lower effect size will require a larger sample size.

One interesting observation from published eQTL studies is that most of the strong eSNPs are located near the TSS with no discernable trend in the 5' or 3' direction[28,39,40]. As a result, most studies consider SNPs within close proximity of the TSS (± 500 kb window) as *cis*-eSNPs. Since the genomic context of most eQTL transcripts are known, statistical adjustment for the actual number of SNPs tested within 500 kb will be more appropriate for *cis*-eSNP discovery. Any SNP outside the *cis*-region is tested as a *trans*-eSNP for a transcript. The molecular biological basis of trans-regulation is less studied; current information suggests that the variants that affect

transcription factors, miRNAs, or long-range chromatin interaction may act as trans-eSNPs. To identify *trans*-eSNPs, the number of tests needed is far greater, and the tests require more stringent significance threshold criteria and a larger sample size. Thus, use of a false discovery rate based on a permutation analysis to correct for multiple testing[34], and considering the correlation among transcript levels and highly correlated SNP structures, are useful approaches to identify this biologically important class of regulatory SNPs.

Several heterogeneous sources of variability hidden in the data may lead to both spurious eSNPs and missed associations in eQTL analyses if not properly addressed. Statistical models that correct for hidden structures within the sample (such as race, admixture, and family relatedness), artifacts in expression data (including batch effects and probe bias), environmental influences, and other known and unknown factors are required to improve sensitivity and interpretability of eQTL analyses[41]. Methods that showed significant usefulness in tackling these confounding factors include Bayesian approaches developed by Stegle *et al*[42] (implemented in probabilistic estimation of expression residuals or probabilistic estimation of expression residuals software), linear mixed-effects model-based approaches developed by Listgarten *et al*[43] (implemented in LMM-EH-PS or Linear Mixed Model-Expression Heterogeneity-Population Structure software), surrogate variable analysis, and inter-sample correlation emended approaches[44,45].

The heavy computational burden involved in eQTL analyses sometimes forces researchers to restrict their analysis to a small subset of selected transcripts and SNPs. Improvement of computational algorithms, parallelization of programs by efficient scripting, and utilization of efficient processing hardware are among many approaches needed to improve scalability and computational efficiency required for eQTL analyses. Implementation of these approaches will enhance discovery by increasing the capacity to utilize the complete data set[46,47].

EQTLS AND DISEASE GENE MAPPING

Molecular and cell biological experiments in model organisms and cells have significantly advanced our understanding about the role of non-coding DNA sequences in genetic regulation, transcriptional circuitry, the transcriptional apparatus, and chromatin regulation. This work has led to new insights into the complex mechanisms involved in dysregulation of gene expression in various human diseases[48]. Recent genome-wide studies in human cells by different international consortia [including ENCyclopedia Of DNA elements (ENCODE)][49] further have improved our mechanistic understanding of the role of DNA sequence variants in quantitative modulation of gene expression[50-52]. eQTL studies have been extensively used to identify genetic regulators involved in natural variation of gene expression[28,37,39] and to understand tissue-specific architecture of genetic regulatory mechanisms[24,30,53-59].

However, an intriguing application of eQTL mapping is the use of eSNP data to interpret disease or disease-related phenotypic association signals, and thereby elucidate specific biological mechanisms underlying the increased genetic risk attributed by the DNA sequence variants. Identification of genetic variants simultaneously associated with disease and eQTLs (in relevant tissue) significantly facilitates identification of potential causal genes. Discovery of genetic variants in *ORMDL3* as a susceptibility factor for childhood-onset asthma[60] and *VNN1* variants that influence high-density lipoprotein cholesterol concentrations[26] are two early examples of the successful implementation of eQTL mapping in disease gene hunting. The review by Cookson *et al*[36] offer a more detailed discussion on those success stories.

Several recent studies have integrated GWAS and eQTL analyses (data generated in different sets of subjects) and have used the overlap of two signals as a tool to interpret GWAS findings. Although this work is a good starting point, we need to be cautious about using the overlap of two statistical signals (eSNP and the disease phenotype-associated SNP/phSNP). Careful thought is required before making a claim of identifying a disease-causing variant. Montgomery and Dermitzakis (2011) described three situations[41] when a coincidence of eQTL and disease phenotype GWA signal may distract from identification of causal variants: (1) eSNP and phSNP are in the same linkage disequilibrium (LD) block but are two different SNPs. This is not considered as exact overlap, and they may tag different causal variants; (2) eSNPs and phSNPs are the same but SNP density differs between the eQTL and GWAS data. Lack of proper resolution in one or both studies may be misleading and will not elucidate the correct functional SNP; and (3) eSNPs may have a pleiotropic effect and may regulate the expression of "gene Y" in "tissue 1", but the same eSNP may regulate the expression of "gene X" in "tissue 2". Thus, if the eQTL study is done in "tissue 1" (a "surrogate" tissue) but not in "tissue 2" (the "disease-relevant" tissue in which the true causal effect is manifested), then despite the overlap of eSNPs and phSNPs, we will incorrectly link "gene Y" to the disease phenotype.

In general, eSNPs that are universal have a stronger effect, but a significant proportion of eSNPs show tissue-specific effects[30,53,54]. However, it is difficult to select "relevant" tissue, or the relevant tissue may not be accessible from human subjects for analysis for many complex diseases. Ongoing efforts of international consortia, including GTEx, to develop multi-tissue eQTL databases (Table 1) is a significant step forward in addressing this limitation[61-64].

Many investigators have developed statistical approaches to formally test the overlap of GWAS and eQTL signals to distinguish accidental colocalization from true sharing of causal variants. The regulatory trait concordance method designed by Nica *et al*[65] accounts for local LD structure and integrates eQTL and GWAS results to reveal the subset of association signals due to *cis*- or *trans*-eQTLs. He *et al*[66] (2013) developed an algo-

Table 1 Selected expression quantitative trait loci databases

Database	Website (URL)	Cell/tissue type	Project
eQTL Browser	http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/	LCL, liver, brain, fibroblast, T-cell	17 projects
Genvar	http://www.sanger.ac.uk/resources/software/genevar/	Adipose, LCL Skin fibroblast from healthy female twins	MuTHER
		LCL from 8 populations	Hapamap3
		Fibroblast, LCL and T-cell from umbilical cord	GenCord
GTEx eQTL Browser	http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi	Multiple tissues including liver, brain regions, LCL	GTEx
PACdb	http://www.pacdb.org/	Gene-drug or GXD eSNPs from LCL model	Dolan and Cox lab
SGR Database	http://systems.genetics.ucla.edu/	22 mouse and several human datasets. Data includes aortic endothelial and smooth muscle, adipose, brain, liver, macrophages and muscle tissue Includes GXE eSNP data from cell experiments	Lusis lab
SCAN	http://www.scandb.org/newinterface/about.html	CEU and YRI LCLs from HapMap	Cox Lab
seeQTL	http://www.bios.unc.edu/research/genomic_software/seeQTL/	HapMap LCLs	

SGR: Systems genetics resource; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes; eSNP: Expression regulatory SNP; LCL: Lymphoblastoid cell lines; GXD: Gene-by-drug interaction; GXE: Gene-by-environment interaction; CEU: HapMap caucasian from CEPH collection; YRI: HapMap African from Yuroba, Nigeria.

rithm named "Sherlock" based on a Bayesian statistical framework to identify potential gene-disease associations by matching genetic signatures of expression (collective information of *cis*- and *trans*-eSNPs) of a gene to that of the disease phenotype by using GWAS data of the disease and the eQTL data of related tissue. These novel approaches are likely to expand our ability to harvest new insights from genetic association studies for disease phenotypes.

T2D-ASSOCIATED VARIANTS ARE ESNPS IN TISSUES IMPORTANT FOR GLUCOSE HOMEOSTASIS

Genome-wide eQTL analyses in transformed lymphocytes (lymphoblastoid cell lines, or LCLs) provided the first evidence that SNPs associated with complex diseases phenotypes are more likely to be eSNPs than minor allele frequency-matched SNPs randomly selected from high-throughput GWAS genotyping platforms. Nicolae *et al*[67] (2010) utilized an Affymetrix GeneChip Human exome 1.0 ST array to generate exon-level expression data of LCLs from 87 Caucasian (CEU) and 89 African (YRI) subjects from the HapMap project. They performed a quantitative-trait transmission disequilibrium test to identify eSNPs from 2 million genotyped SNPs. A study by Nica *et al*[65] (2010) utilized an Illumina Sentrix WG-6-V2 whole-genome expression array to generate total transcript-level expression data of LCLs from 109 unrelated CEU subjects (from the HapMap 3 project) and performed Spearman rank correlation analysis to identify eSNPs from 1186075 genotyped SNPs. Key findings from these studies[65] include: (1) SNPs reproducibly associated with complex human traits are likely to be eSNPs; (2) Enrichment of complex trait GWAS-implicated SNPs are more evident among *cis*-eSNPs but not among *trans*-eSNPs; and (3) eSNPs discovered in LCLs are more strongly enriched for SNPs associated with immunity-related conditions (*e.g.*, Crohn's disease, type 1 diabetes, rheumatoid arthritis), but such enrichment was not observed for metabolic disorders (*e.g.*, T2D and coronary artery disease). These studies indicate that eQTL studies using surrogate tissue samples may be helpful for some diseases. However, understanding the functional role of T2D-associated SNPs will probably require expansion of eQTL studies into tissues more relevant for T2D pathophysiology. These studies also had significantly lower power to identify *trans*-eQTLs due to comparatively small sample sizes, and will require reevaluation of the role of *trans*-eSNPs in larger sample sets.

Zhong *et al*[68] (2010) used genetics of gene expression (GGE) analysis in tissues from two cohorts of human subjects (Cohort 1: liver-specific GGE cohort with post mortem liver samples from 427 subjects; Cohort 2: liver, subcutaneous adipose and omental adipose from 922 subjects who had Roux-en-Y gastric bypass surgery). They identified 18785 unique eSNPs in the combined set of data. They found 2189, 2286, and 1999 eSNPs specific to liver, omental adipose, and subcutaneous adipose, respectively. However, they also noticed that 72% of *cis*-eSNPs identified in liver, 79% of those found in omental adipose and 80.5% from subcutaneous tissue were also found in the other two tissues. Given the metabolic relevance of these tissues, they further interrogated data from three large-scale T2D GWAS datasets to test whether the set of eSNPs were more likely to be associated with T2D compared to randomly selected SNPs. These tissue eSNPs showed a significant enrichment of T2D-associated SNPs. For example, in the DIAGRAM (DIAbetes Genetics Replication and Meta-analysis) GWAS data set, 7.34% of the eSNPs showed a significant association with T2D ($P < 0.05$) compared to an average of 6.12% SNPs in the random sets, representing a modest 1.20-fold enrichment for SNPs in the eSNP (or SNP in LD at $r^2 > 0.89$) set over the random sets (p-enrichment = 1.33×10^{-9})[68]. In that study, omental adipose tissue eSNPs also showed further significant enrichment when restricted to adipose

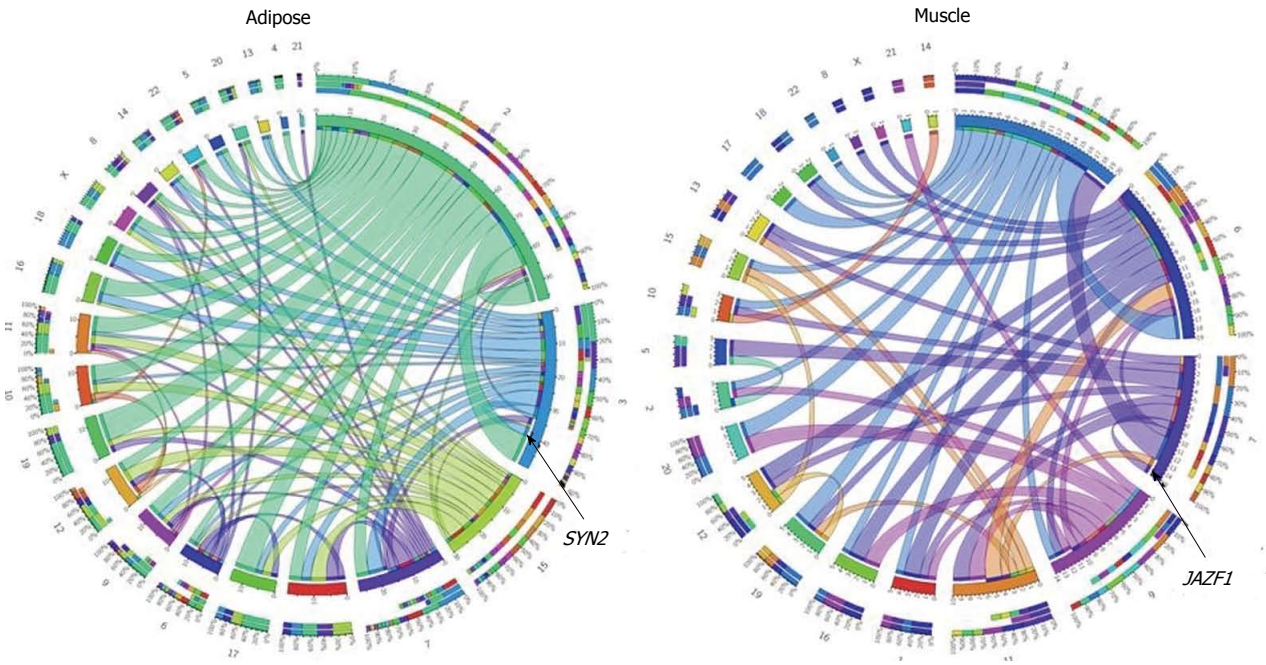

Figure 2 Type 2 diabetes or glucose homeostasis traits associated variants are expression regulatory SNP. We tested *Cis* and *Trans* regulatory role of 68 SNPs that showed reproducible associations with T2D or Glucose homeostasis traits[72]. At a threshold of $P < 0.0001$, 25 and 19 of these SNPs in adipose and muscle, respectively, showed association with expression of a *cis*- or trans-transcript. This figure represents a CIRCOS plot of eQTL and eSNP chromosomal location relationships, indicating the predominance of *trans*-regulation among 183 and 62 significant ($P < 0.0001$) eQTL-eSNPs associations in adipose and muscle respectively. Rare *cis*-regulation (*SYN2* in adipose and *JAZF1* in muscle) is marked. eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

expression network genes differentially expressed with T2D. Thus, these studies support the notion that T2D-associated SNPs may modulate expression of transcripts in tissues relevant for glucose homeostasis.

Fu *et al*[53] (2012) analyzed eQTLs in blood ($n = 1240$) and other tissues (liver, $n = 62$; muscle, $n = 62$; subcutaneous adipose, $n = 83$; and visceral adipose, $n = 77$); out of 1954 SNPs associated with complex disease traits from a GWAS catalogue, 907 were *cis*-eSNPs. However, 28.7% of these trait-associated *cis*-eSNPs showed a tissue-specific (in blood versus other tissue) and discordant effect on gene expression. The discordant effect includes tissue-specific regulation, alternative regulation by different eSNPs, different effect size and, in a few cases, opposite allelic direction. The study also showed that SNPs associated with complex traits are more likely ($P = 2.6 \times 10^{-10}$) to exert a tissue- specific effect on gene expression[53]. No comparisons were made between other tissues due to small sample size. This study indicates that use of tissues in eQTL analysis may have implications for inferring transcriptional effects of SNPs, especially for the complex disease susceptibility variants.

This work also emphasizes the importance of investigating disease-relevant tissue for characterizing functional effects of T2D and other disease-associated variants. However, it is difficult to determine "relevant tissue" even for diseases with known pathophysiology. T2D is clearly of polygenic etiology, and relevant tissue could be distinct for genes involved. Moreover, gene expression is regulated by environmental (*e.g.*, diet), epigenetic, and other unknown factors, and eQTL discovery from tissue samples may be affected by the physiological state of the donors[41]. For example, profound hyperglycemia and dyslipidemia observed in T2D subjects will modulate and even may mask primary causal changes in genetic regulatory networks. Thus, multi-tissue eQTL analysis in physiologically characterized individuals could be a safe option to scrutinize the circularity of cause and effect in genetic regulatory signals, and holds the promise to offer insights into the novel mechanisms driving genetic susceptibility to T2D.

Most initial eQTL studies seeking to identify a regulatory role for T2D-associated SNPs have focused on *cis*-eQTLs. However, studies by Voight *et al*[69] (in adipose, $n = 603$; and blood, $n = 745$ subjects) and our laboratory (in adipose and muscle of 168 non-diabetic subjects who were physiologically evaluated) showed that only a few top T2D GWAS-identified signals can be explained as *cis*-eQTLs, and T2D-associated non-coding SNPs are less likely to regulate expression of the closest gene[70]. Results were similar in an eQTL analysis that used human islet cells from 63 cadaver donors[71]. A genome- wide study by our laboratory[72] in adipose and muscle tissue of 62 subjects (31 insulin- resistant and 31 insulin-sensitive subjects matched for BMI) showed that at a less stringent threshold ($P < 0.0001$), among 68 well-replicated T2D/glucose homeostasis-associated SNPs, 25 and 19 of them were eSNPs in adipose and muscle, respectively (Figure 2). However, after stringent (Bonferroni) correction, only SNP rs13081389 was a *cis*-eSNP for the *SYN2* gene in adipose ($P < 4.7 \times 10^{-8}$, 15507 expressed transcripts were tested in adipose). Interestingly, these 68 SNPs showed

significant enrichment for *trans*-eSNPs in adipose and muscle, but not in LCLs[72]. Many of these *trans*-eSNPs show associations with expression of ⩾ 10 transcripts and may be a "master regulator". Expanding this search for the top 1000 T2D-associated SNPs from a Wellcome Trust Case Control study also confirmed the *trans*/distal regulatory SNPs[72]. We also showed that replicated T2D- and glucose homeostasis-associated SNPs are enriched for *trans*-eQTLs for transcripts differentially expressed between insulin-resistant and insulin-sensitive people[72]. A recent eQTL study using a large cohort of blood samples also supported the *trans*-regulatory role of 233 complex trait-associated SNPs[73]. Thus, the genetic regulatory architecture of T2D is complex, tissue-specific, and likely extends beyond the *cis*-regulatory mechanism.

EQTL ANALYSIS FOR PRIORITIZING T2D-ASSOCIATED VARIANTS TO IDENTIFY NOVEL CANDIDATE GENES

The multiple testing corrections utilized in genome-wide statistical analyses allow detection of only the strongest effects and penalize weaker associations that may be biologically meaningful[74]. Investigators have implemented several approaches to prioritize T2D association signals from large GWAS datasets to identify biological mechanisms responsible for genetic predisposition. One common approach includes selection of genes close to T2D GWAS-implicated SNPs and shows differential expression in T2D subjects compared to normoglycemic subjects (or in animal models of T2D). This approach is based on the idea that T2D-associated variants may modulate the expression of nearby genes in tissues important for glucose homeostasis. Parikh *et al*[75] used publicly available expression microarray data from different tissues (pancreas, adipose, muscle, and liver from T2D patients and rat models of T2D) to prioritize among the 275 genes located near 1170 T2D GWAS-implicated SNPs. A recent study by Taneera *et al*[71] used expression profiling of human pancreatic islet cells for functional prioritization of genes in the vicinity of 47 T2D-associated SNPs. However, available data from several human tissue eQTL analyses indicate that only a few T2D-associated SNP act as *cis*-eSNPs, and no enrichment of differentially expressed genes was observed around T2D GWAS-implicated variants[72]. Thus, a logical alternative for prioritizing T2D-associated variants is to utilize a reverse genetics approach and restrict the genetic search space to the subset of variants that are eSNPs in relevant tissues. These eSNPs are statistically associated with expression of transcript and thus have a strong possibility of being a "key driver" in perturbing gene-expression regulatory networks.

Selecting the genes based on eSNPs among those also associated with T2D in large GWAS datasets will prioritize genes with a significantly high chance of being causally involved with susceptibility to T2D, and thus may be helpful in identifying additional genetic susceptibility loci from GWAS datasets. A genome-wide analysis of adipose tissue transcriptomes from 62 insulin-resistant and -sensitive subjects identified 172 differentially expressed transcripts[76]. We checked adipose eQTL data from the MuTHER study[55] to find eSNPs of these differentially expressed transcripts. We further mined the DIAGRAM GWA meta-analysis results[13] for association of these eSNPs with T2D. This analysis[77] identified that the strongest *cis*-eSNP (rs11037579, $P = 4.21 \times 10^{-6}$) for the *HSD17B12* in adipose tissue was also associated with T2D [$P = 3.80 \times 10^{-4}$, OR = 1.06 (95%CI: 1.03-1.1)]. Individuals carrying the T2D risk allele T for the intronic SNP rs11037579 had lower expression of *HSD17B12* in adipose tissue. This result corroborates the finding that *HSD17B12* expression is downregulated in the adipose tissue of insulin-resistant subjects. The *HSD17B12* gene codes a bifunctional enzyme involved in the biosynthesis of estradiol and the elongation of very long chain fatty acids. Several variants within ± 500 kb of this gene are eSNPs (including a 3'UTR SNP rs1061810) in adipose, LCL, and other tissues, and show an association with T2D (although below the genome-wide threshold) (Figure 3). Further functional studies will be required to identify true causal SNPs. However, this integrative approach demonstrates the validity of such an approach in prioritizing novel T2D susceptibility loci. In fact, two recent integrative genomic studies showed that eSNPs for *PFKM* (SNP rs11168327) gene in muscle and *ARAP1* (SNP rs11603334) gene in pancreatic beta cell are associated with T2D[78,79].

EQTL AND BIOLOGICAL NETWORK ANALYSIS TO IDENTIFY ETHNIC-SPECIFIC GENES FOR T2D:

Age-standardized prevalence of T2D varies among ethnic and racial groups[14,80]. T2D is almost twice as prevalent in adult non-Hispanic African Americans (14.9%) in the United States compared to European Americans (7.6%)[81]. Yet only a few of the associated T2D-loci - identified primarily in European- or Asian-derived populations - are replicated in African American, Hispanics, and Native Americans[14,16,82-84]. Intriguingly, studies have identified distinctive physiologic features of glucose homeostasis in African Americans and Hispanics[85-87]. Compared to non-Hispanic Caucasians matched on age, gender, and BMI, African Americans are more insulin-resistant (lower S_I), but show a greater acute insulin response to intravenous glucose (AIR_G) and a higher disposition index ($DI = S_I \times AIR_G$). A genetic basis for these physiological differences seems likely, but remains unidentified.

Published studies of expression across ethnic groups (mostly restricted to lymphocytes or HapMap LCLs) showed distinct ethnic-specific expression[37,88-90]. Zhang *et al*[90] (2008) reported differential expression of up to 67% of transcripts between LCLs from subjects of European (CEU) and African (YRI) descent, with enrich-

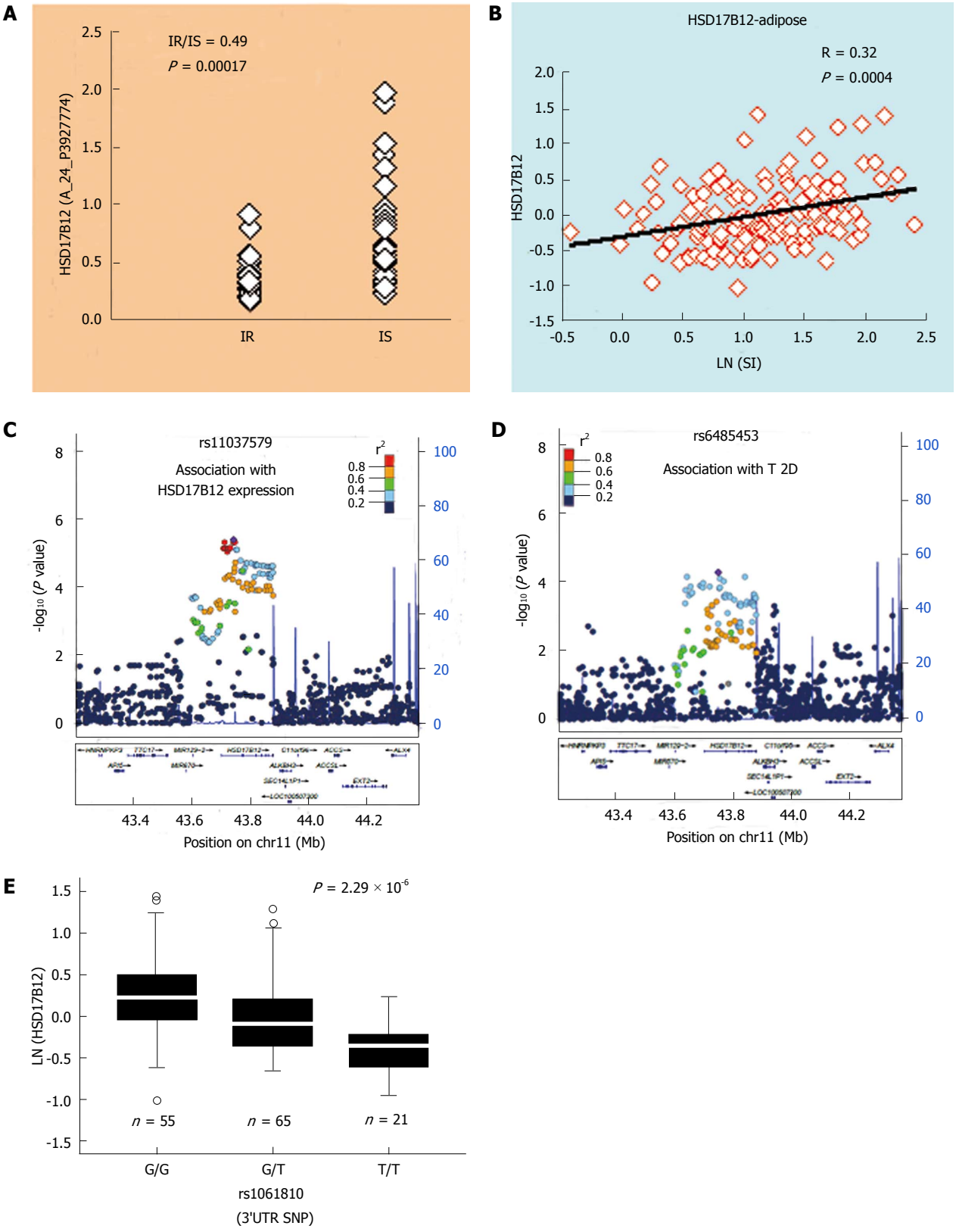

Figure 3 Prioritizing type 2 diabetes-associated variants by expression quantitative trait loci analysis: An example. *HSD17B12* is one of 172 genes differentially expressed in adipose tissue of insulin-resistant (IR, $n = 31$) *vs* insulin-sensitive (IS, $n = 31$) subjects in a genome-wide study (A) by Elbein *et al*[76]. Its expression in subcutaneous adipose of non-diabetic subjects ($n = 141$) also shows a significant correlation (B) with insulin sensitivity (SI). Strongest *cis*-eSNP for adipose tissue (C) expression of HSD17B12 (in adipose eQTL from the MuTHER project)[55] is also associated with T2D (D) in a large GWAS meta-analysis (in DIAGRAM.v3 data from 12171 T2D and 56862 controls)[13]. This locus also includes a 3'UTR SNP rs1061810 that shows association (E) with T2D and expression of HSD17B12 (in qRT-PCR analysis in adipose tissue from 141 non-diabetic subjects). eSNP: Expression regulatory SNP; eQTL: Expression quantitative trait loci; T2D: Type 2 diabetes.

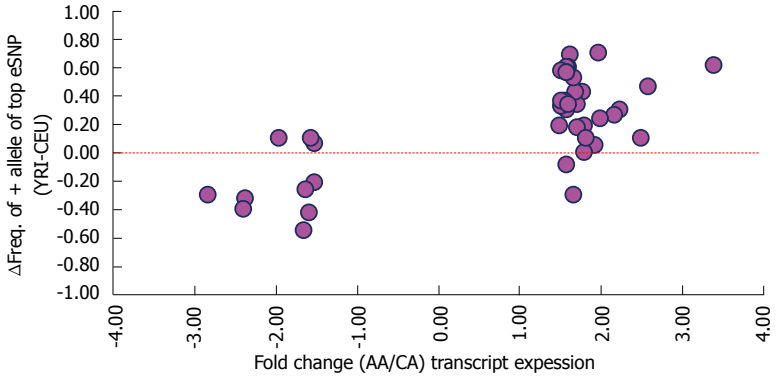

Figure 4 Population differences in expression of transcripts in adipose tissue is accounted for by the effect allele frequency difference of expression regulatory SNPs among racial groups. X axis: Fold change in average expression of 41 transcripts between African-American (AA, *n* = 37) and Caucasian (CA, *n* = 99) subjects. Y axis: Differences in strongest eSNP allele frequency of these transcripts between HapMap subjects of Caucasian (CEU) and African (YRI) ancestry for alleles associated with higher expression. *e*SNP: Expression regulatory SNP.

ment of ribosome biogenesis, antimicrobial response and cell-cell adhesion. Spielman *et al*[37] (2007) attributed the 1097 genes that differed between CEU and Asian (CHB) LCL samples to eSNP frequency. Our comparison of genome-wide expression profiles (using an Agilent 44K expression array) from adipose and muscle tissue of non-diabetic Caucasians (n = 40) and African Americans (n = 22) identified transcripts associated with insulin sensitivity (S_I), many of which (*e.g.*, *CLIC6*, *HSD11B1*, *SERPINA3*, *THBS1*, *TMEM135* and *TNMD* in Adipose) show distinct ethnic-specific expression[76].

Comparison of adipose tissue expression data between Caucasians and African Americans in a larger cohort (using an Illumina –HT12.V4 array for 99 Caucasians and 37 African Americans) identified 117 differentially expressed (fold change $\geqslant$ 1.5 and false discovery rate $\leqslant$ 5%) transcripts[91]. By mining adipose tissue eQTL data from the MuTHER project[55], we found that about 35% of these differentially expressed transcripts are strongly modulated ($P < 1 \times 10^{-5}$) by *cis*-eSNPs in adipose tissue. In line with the findings by Spielman *et al*[37] (2007) in LCL, we also found that in adipose tissue, the degree of differential expression (fold change African Americans/Caucasians) shows strong concordance with the difference in the effect allele frequency of top *cis*-eSNPs (Figure 4) between HapMap African (YRI) and Caucasian (CEU) subjects.

These studies suggest that the distinct genetic architecture of eSNPs determines the ethnic-specific expression profile in tissues important for glucose homeostasis. Ethnic-specific derangements of gene expression networks in tissues involved in glucose homeostasis may explain distinctive physiologic effects, including differences in insulin action and secretion between ethnic and racial groups. Perturbation of gene expression networks associated with early pathophysiologic events (including insulin resistance) is driven by regulatory variants (eSNPs). The distinct genetic architecture of these variants (including linkage disequilibrium and allele frequency) may determine their ethnic-specific (or predominant) effect on expression and T2D susceptibility. Thus, integration of genome-wide expression analysis and eQTL analyses may be a useful approach to identify the primary genetic factors for ethnic-specific susceptibility to T2D.

Expression of transcripts involved in the same biological function tend to be co-regulated by similar factors (genetic or environmental) and can be identified as distinct network modules, where genes within a module are more highly interconnected (correlated) with each other than genes in other modules. Statistical approaches like weighted gene co-expression network analysis (WGCNA software package developed in "R" programming environment implements this analytical method) are useful for identifying modular structures of the co-expression networks[92,93] in tissues important for glucose homeostasis. Evaluation of the correlation of each module eigengene with the S_I and other T2D-related metabolic phenotypes, and determination of the preservation of these modules between ethnic groups based on observed network density and connectivity, will identify molecular processes or molecular interaction structures associated with phenotypes that undergo ethnic-specific reconfiguration by genetic or non-genetic causal regulators.

Several recently developed statistical metrics[94,95], including modular differential connectivity, offer powerful tools to identify the modules with significant ethnic-specific changes in interaction strength. The eSNPs are causal variants (or in linkage disequilibrium with causal variants) that regulate the expression level of neighboring (or distal) genes. Thus, eSNPs serve as a primary source of natural perturbation to infer causal relationships among and between genes in gene-expression networks[96]. The distinct allelic architecture of these SNPs may determine ethnic-specific modular differential connectivity. Genes with eSNPs can be considered as "parent nodes" in expression networks. This information is used as a "structure prior" in the network reconstruction analysis to orient the edges of the networks. Reconstructing ethnic-specific networks by utilizing different causality modeling methods, including Bayesian network reconstruction approaches, may identify key causal regulators of these networks[97,98]. Thus, a multiscale biological network analysis that utilizes eQTL information to distinguish causal from correlated disease effects is a novel approach to understand how causal regulators propagate their effects in mediating ethnic-specific susceptibility to disease.

A similar approach was used recently to identify genetic factors in animal models of diabetes and other complex human diseases, including Alzheimer's disease[95]. A study by Zhong *et al*[68] (2010) in adipose tissue of

C57BL/6-ob/ob × BTBR-ob/ob mice F2 progeny identified a strong causal subnetwork for T2D traits (called the "purple" module, enriched for genes involved in plasma glucose and insulin levels). They found that 37 eSNPs of genes in this module showed significant association with T2D in a GWAS report. Through additional prioritization steps and subsequent function validation studies, they identified mallic enzyme (*ME1*) as a key causal gene in this T2D subnetwork. A strong *cis*-eSNP of *ME1* was associated with T2D. Future applications of such integrative genomic strategies in T2D or related disorders in human populations may prove insightful.

EQTL ANALYSES TO IDENTIFY GENE-ENVIRONMENT INTERACTIONS RELEVANT FOR T2D

As discussed above, GWAS have identified DNA sequence variants in the susceptibility to T2D, but these variants account for only a part of the estimated heritability[13,14]. Interactions between sequence variants and environmental stimuli are a logical step in better understanding the development of T2D. Thus, some of the missing heritability for T2D susceptibility may be explained by studies of the interaction between environmental factors and genetic variants or gene-environment (GXE) interactions[99]. Modeling GXE interactions in clinical or epidemiological settings is challenging and costly, due to few validated tools for assessing exposure (including dietary exposure), the need for large sample sizes, and the heterogeneity of exposures in populations[100-103]. Environmental factors usually influence insulin resistance and T2D risk over long periods of time; thus, accurate assessment of long-term exposure is needed to identify GXE interactions. A recent series of studies by Patel *et al*[104-106] utilized data resources from the National Health and Nutritional Examination Survey and integrated GWAS and environment-wide association studies to identify environmental factors, genetic factors, and GXE interactions involved in T2D susceptibility. However, they noted several significant limitations of such epidemiological approaches in adequately addressing influence of genetic variations on differences in environmental response in human populations.

Environmental factors, including diet and derived metabolites, can influence phenotypes by modulating gene expression in several ways. Variations in responses to environmental factors among individuals, and how these responses predispose to metabolic and other disorders, have been recognized[107]. Genetic variants modulate the environmental factor-mediated transcriptional response, which in turn dictates cellular response and may explain variability in metabolic responses to those factors[99]. Such dependency on external conditions or GXE interactions has been reported for genetic effects on gene expression in different organisms[108-110]. Transcripts responsive to environmental perturbation factors may manifest as eQTLs and are modulated by *cis*- and *trans*-eSNPs. A subset of these eSNPs associated with T2D, obesity, and/or glucose homeostasis traits may thus exhibit distinctive patterns of GXE eSNPs. Thus, identifying environmental factors that modulate insulin sensitivity and other early pathophysiological manifestations of T2D and its integration into eQTL analyses will further improve the power to construct causal gene regulatory networks involved in T2D susceptibility.

A few recent studies implemented a novel "cellular genomics" approach[111] to elucidate genetic controls on GXE interactions, critical to understanding the pathophysiology of complex diseases. In this novel paradigm, researchers analyzed the molecular consequences of genetic variants to assess interactions with environmental factors *via* quantification of processes (like gene expression) in cells from human subjects grown in uniform culture conditions. This concept is illustrated in Figure 5. Utilizing transformed lymphocytes, the studies examined genetic control in response to radiation, chemotherapeutic drugs, and hormones (glucocorticoids)[112-114]. Two similar studies in primary human cells mapped genetic regulators responding to growth factors (BMP-2), hormones (dexamethasone), cytokines (prostaglandin E2 in human osteoblasts), and oxidized low density lipoprotein (in human aortic endothelial cells)[115,116]. Despite the encouraging success of these studies, no studies so far have evaluated GXE interactions with a cellular genomic model relevant to T2D and related metabolic disorders. Although this model may miss some whole organism-level complexity[117] of T2D pathogenesis (which involves multiple tissues), it does represent an innovative approach by going from cellular to organismal phenotype analysis for identification of function of genetic variants involved in T2D susceptibility. Mapping GXE eSNPs for function-based prioritization of T2D and related metabolic disease-associated SNPs is a critical step towards designing efficacious strategies to reduce the public health burden of common metabolic disorders triggered by increased exposure to dietary and other environmental factors.

EQTL AND PHARMACOGENOMIC STUDIES FOR T2D

Several classes of anti-diabetic medications are used for the treatment of T2D[118]. Pharmacogenomic studies reviewing the role of genetic variants on drug responses (including adverse drug reactions) have yielded significant findings, including novel disease mechanisms for several complex diseases[119]. But a similar success for T2D has not been achieved[5,120]. Pharmacological interventions using peroxisomal proliferator activated receptor gamma (PPARγ) agonists like pioglitazone improve insulin sensitivity and can reduce the risk of progression to T2D[121]. However, approximately 25% of patients do not respond adequately to PPARγ agonists[122]. Genome-wide transcriptomic analysis by our laboratory showed significant inter-individual variability in gene-expression response

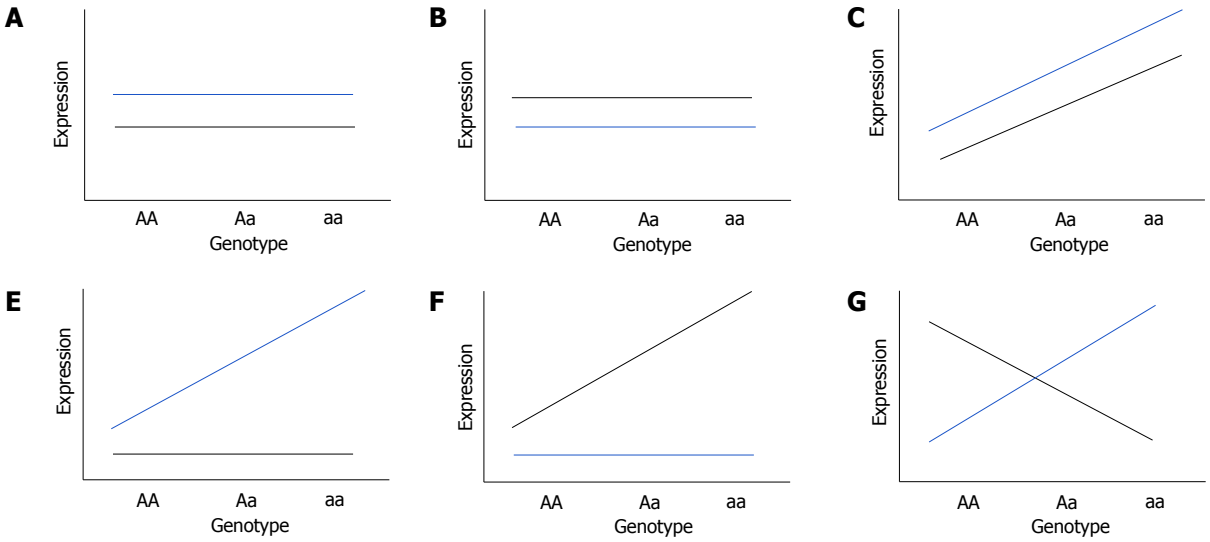

Figure 5 Types of gene-by-environment interactions in cellular genomic models to study gene-by-environment expression quantitative trait locis. Cells from a cohort of subjects are grown in pairs under uniform *in vitro* treated and untreated conditions to study environment-dependent or -independent effects of genotype on expression of transcripts (a quantitative trait). β1 and β2 are genotype effects on transcript expression under treated and control conditions, respectively. Different models of gene-by-environment (GXE) includes Null model: β1 = β2 = 0 (A and B); No-interaction eQTL model: β1 = β2 ≠ 0 (C); Treated-only expression quantitative trait loci model: β1 ≠ 0 and β2 = 0 (D); Control-only eQTL model: β1 = 0 and β2 ≠ 0 (E); and General interaction eQTL model: β1 ≠ 0 and β2 ≠ 0 but β1 ≠ β2 (F). Black line indicates expression in cells under control condition (untreated) while blue line indicates expression in environmental/dietary factor treated cells.

after pioglitazone treatment in people with impaired glucose tolerance[123]. However, little is known about the genetic architecture of variation in pioglitazone-mediated transcriptional response in human populations. Identifying the genetic variations that interact with pharmacological treatments like PPARγ agonists is of high clinical interest. eSNPs may modulate the expression of key transcripts in response to anti-diabetic drugs in target tissues and can explain the interindividual variability in treatment outcome[124,125]. Identifying genetic (and epigenetic) variants that modulate the pharmacological treatment-mediated transcriptional response, which in turn dictates the treatment outcome in T2D, is an open area of research. A novel approach that systematically characterizes the set of eSNPs involved in anti-diabetic medicine-mediated transcriptional modulation (gene-drug interaction eSNPs, or GXD eSNPs) in tissues relevant to glucose homeostasis will be useful in stratifying populations in efficacy studies, to improve the quality of clinical decision-making and treatment options for T2D.

FINDING EQTLS: END FOR A NEW BEGINNING

eQTL analyses provide statistical evidence for genotype-dependent variations in transcript abundance and should be considered a starting point for investigating the effects of DNA polymorphisms at the molecular level[34]. Transcript abundance depends on a dynamic relationship between transcript synthesis, stability, and degradation[48]. Thus, DNA polymorphisms may affect transcript abundance by several known and unknown mechanisms. Studies in human subjects have shown that sequence-specific regulation of mRNA expression is mediated by several molecular mechanisms, including allelic variability in transcription factor binding, chromatin remodeling, changes in DNase I hypersensitivity by histone methylation and acetylation, interaction between chromatin segments, alteration of splicing, sequence-dependent allele-specific DNA methylation, alteration of miRNA synthesis, and miRNA target binding[50-52,126-130]. GWAS-implicated variants for complex diseases are enriched in non-coding functional domains of the genome, including sequences involved in chromatin remodeling[131-133]. Many transcripts that show strong co-expression and *cis*-eSNPs for one transcript may appear as trans-eSNPs for a co-regulated transcript located in other chromosomes. Thus, a functional role of prioritized *cis*- and *trans*-eSNPs needs to be validated by appropriate molecular experiments to distinguish causal from correlative effects[134-136]. Studies have used allelic expression imbalance analysis, electrophoretic mobility shift assays, and transient transfection based luciferase reporter assays[56,137-141] to identify the molecular effects of genetic variants (*cis*-eSNPs) on gene expression; however, high-throughput methods are needed to validate in parallel the large number of findings from genomic studies[134,135,142]. Several novel high-throughput methods, including massively parallel reporter assays and massively parallel functional dissection, are now available to show evidence of causality for regulatory variants[143-146]. Functional relevance of the candidate eQTL transcripts in T2D pathophysiology also need to be validated by demonstrating their effects upon experimental up- or down-regulation in *in vitro* or *in vivo* experimental models[147,148].

In summary, many factors (including genetic, epigenetic and environmental factors) affect susceptibility to T2D. Instead of investigating different sources of varia-

tion in isolation, an integrative functional omics paradigm that traces early molecular changes through layers of biological information, including eQTLs, promises to be a useful approach[136]. Such an approach will promote optimal understanding of the etiology of T2D and lead to the identification of ethnic-specific primary causal variants. Ultimately, the knowledge gained from studies using these approaches can be used to build better classifiers of T2D risk than those based on DNA sequence variants alone.

ACKNOWLEDGMENTS

We thank Karen Klein (Translational Science Institute, Wake Forest University Health Sciences) and Ethel Kouba (Internal Medicine, Endocrinology) for critical reading and editing of our manuscript. We dedicate this article to the memory of Dr. Steven C. Elbein.

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P- Reviewers: Cai H, Guneli EM, Harwood HJ, Mansour AA, Vorobjova T **S- Editor**: Wen LL **L- Editor**: A
E- Editor: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Recent advances in the molecular genetics of type 2 diabetes mellitus

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Received: November 19, 2013 Revised: December 28, 2013
Accepted: January 17, 2014
Published online: April 15, 2014

Abstract

Type 2 diabetes mellitus (T2DM) is a complex disease in which both genetic and environmental factors interact in determining impaired β-cell insulin secretion and peripheral insulin resistance. Insulin resistance in muscle, liver and fat is a prominent feature of most patients with T2DM and obesity, resulting in a reduced response of these tissues to insulin. Considerable evidence has been accumulated to indicate that heredity is a major determinant of insulin resistance and T2DM. It is believed that, among individuals destined to develop T2DM, hyperinsulinemia is the mechanism by which the pancreatic β-cell initially compensates for deteriorating peripheral insulin sensitivity, thus ensuring normal glucose tolerance. Most of these people will develop T2DM when β-cells fail to compensate. Despite the progress achieved in this field in recent years, the genetic causes of insulin resistance and T2DM remain elusive. Candidate gene association, linkage and genome-wide association studies have highlighted the role of genetic factors in the development of T2DM. Using these strategies, a large number of variants have been identified in many of these genes, most of which may influence both hepatic and peripheral insulin resistance, adipogenesis and β-cell mass and function. Recently, a new gene has been identified by our research group, the *HMGA1* gene, whose loss of function can greatly raise the risk of developing T2DM in humans and mice. Functional genetic variants of the *HMGA1* gene have been associated with insulin resistance syndromes among white Europeans, Chinese individuals and Americans of Hispanic ancestry. These findings may represent new ways to improve or even prevent T2DM.

Key words: Genome-wide association study; Candidate gene; Genetic variants; High-mobility group A1; Insulin resistant diabetes

Core tip: Despite the progress in clinical and laboratory investigations, the fundamental cause of type 2 diabetes mellitus (T2DM) remains uncertain. Candidate gene, linkage and genome-wide association studies have highlighted the role of genetics in the development of T2DM. Using these strategies, a large number of variants have been identified in many genes, most of which may influence an individual's risk of developing T2DM. In this review, we compile information on genetic factors that influence the risk of T2DM. In addition, we discuss the results from recent studies on the role of HMGA1 on the issue, which might be important for future breakthroughs in this field.

Original sources: Brunetti A, Chiefari E, Foti D. Recent advances in the molecular genetics of type 2 diabetes mellitus. *World J Diabetes* 2014; 5(2): 128-140 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/128.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.128

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic endocrine and metabolic disease that is often associated with be-

ing overweight or frank obesity. It affects millions of people worldwide, with a rapidly increasing incidence and prevalence[1,2]. The latest estimate from the International Diabetes Federation (http://www.idf.org) is equivalent to a global prevalence rate of 8.4% of the adult population, while worldwide diabetes cases hit a new record at 382 million in 2013. Among the determinants of this steadily increasing trend is the combination of genetic and environmental factors responsible for either a positive energy balance resulting in body fat accumulation and weight gain and/or a reduced energy expenditure from a reduction in physical activity and a sedentary lifestyle. Despite extensive attempts at clinical management of T2DM, many diabetic patients will develop a wide variety of long-term complications, including retinopathy, nephropathy and cardiovascular diseases that are among the most frequent causes of morbidity and mortality in affected people, whose effective prevention and treatment require enormous efforts and funding[3]. Typically, T2DM is presented as a common, heterogeneous, complex disease in which both predisposing genetic factors and precipitating environmental factors interact together and cause hyperglycemia, which constitutes the primary hallmark of T2DM[4,5]. Although still poorly understood, the role of genetics in T2DM is well documented. This is supported by a series of evidence, including the strong familial aggregation of the disease, in which the risk of developing T2DM is 40% for those who have an affected parent (higher if the mother rather than the father) and 70% if both parents are diabetics[6]. The highest risk in first-degree relatives, compared to the general population, persists even after removal from the family of origin, for example, as a result of adoption. Furthermore, in identical monozygotic twins (with identical genetic makeup), the concordance rate for the disease approaches 100%, much higher than that seen in non-identical (dizygotic) twins or among siblings[7]. Genetic predisposition in T2DM is also supported by the observation that differences in disease prevalence rates exist among populations, even after migration of entire ethnic groups to another country, thus independent from the environmental influences[8].

On the other hand, the role of environmental factors in influencing susceptibility to T2DM is equally well known. Among these factors are increased caloric intake and a sedentary lifestyle, two conditions common in populations with a higher standard of living and a more westernized lifestyle, responsible for most of the excess weight and obesity in the modern adult's life[9]. The spread of the western way of life in developing countries also explains the epidemic explosion of the disease[1,2], whereas the existing epidemiological data show that the spatial and temporal distribution of T2DM in the geographical areas examined is comparable to the trend of being overweight and obesity[10]. The excess weight causes insulin resistance, which represents the initial step in the natural history of T2DM. Initially, in individuals destined to become diabetic, pancreatic β-cells compensate for the insulin resistance by secreting increased levels of insulin, thus ensuring post-prandial euglycemia[11]. Hyperglycemia in insulin resistant subjects develops later when the β-cells fail to compensate. Thus, from a pathophysiological standpoint, T2DM is characterized by a combination of peripheral insulin resistance and inadequate insulin secretion by the pancreatic β-cells. As supported by numerous studies in the literature[12,13], both defects are the result of a complex interaction between genetic and environmental factors (Figure 1), including chemical agents (calcium and zinc ions) and polluting organic substances that are suspected to play a role in amyloid fiber formation in pancreatic β-cells, thus contributing to the pathology of T2DM[14-17]. The involvement in the pathogenesis of T2DM of multiple genes that interact with each other in an epistatic manner may explain why, despite the enormous efforts made to date, the identification of genetic determinants responsible for an increased susceptibility to T2DM still remains unsolved[18,19].

The present review aims to give an overview of the recent findings in this context. We also discuss the results from some recent studies which might be important for future breakthroughs in this field.

GENETIC STUDIES

Over the past few years, various international research centers have been involved in the study and identification of genes predisposing to T2DM using various methods of investigation. Linkage analysis was used to identify potential genes associated with the disease, starting from the analysis of families and then studying a small number of individuals genetically related to each other. Genotyping for genetic markers in family members with and without T2DM has allowed the identification of DNA regions containing loci associated with disease risk. Thanks to this method, the association of T2DM with the calpain-10 (*CAPN10*) gene[20] was initially identified and later its association with the transcription factor 7-like 2 (*TCF7L2*) gene[21], whose genetic variants in affected individuals increase the risk of diabetes approximately 1.5 times[19].

Another approach used was to search for genetic variants within functional candidate genes encoding for protein(s) with important implications for glucose homeostasis and positional candidate genes that have a genetic association on the basis of a previous linkage study. This experimental strategy is applied to population studies rather than studies of families. Association studies of functional candidate genes represent one of the most powerful approaches as the pathogenetic mechanism of any genetic abnormality would be easily explained. The limit of this strategy, however, is constituted by the fact that it allows focused attention on a single gene at a time. Although many studies have reported associations of functional and positional candidate genes with T2DM, only some of these showed a significant and reproducible association with the disease (Table 1).

From 2007 onwards, the list of candidate genes has grown considerably, largely due to genome-wide association studies (GWAS), a technique commonly used to

Table 1 Type 2 diabetes mellitus susceptibility genes

Gene	Chr	Odds ratio	RAF	Study	Function and probable mechanism	Ref
ADAMTS9	3	1.09-1.05	0.68-0.81	MA	Metalloproteinase/Insulin action	[22–24]
ADCY5	3	1.12	0.78	MA	Adenylyl cyclases/Insulin action	[25]
ANK1	8	1.09	0.76	MA, CC	Cell stability/β-cell function	[26–28]
ANKRD55	5	1.08	0.7	MA, CC	Insulin action	[26,27]
ANKS1A	6	1.11	0.91	GWAS	Pathway regulator/Unknown	[29]
ARAP1	11	1.08-1.14	0.81-0.88	GWAS, MA	Actin cytoskeleton modulator/β-cell function	[22,24]
BCAR1	16	1.12	0.89	MA, CC	Docking protein/β-cell function	[26,27]
BCL2	18	1.09	0.64	GWAS	Cell death regulator/Unknown	[24]
BCL11A	2	1.08-1.09	0.46	MA	Zinc finger/β-cell function	[22]
CAMK1D	10	1.07-1.11	0.18	LA, MA	Protein kinase/β-cell function	[22–24]
CDC123					Mitotic protein/β-cell function	
CAPN10	2	1.09-1.18	0.73-0.96	MA	Calpain cysteine protease/Insulin action	[30–33]
CDKAL1	6	1.10-1.20	0.27-0.31	GWAS, MA	β-cell function	[24,34–36]
CDKN2A	9	1.19-1.20	0.82-0.83	GWAS	Cyclin-dependent kinase inhibitor/β-cell function	[24,34,35]
CDKN2B						
CENTD2	11	1.08-1.13	0.81-0.88	GWAS	β-cell function	[22,24]
CHCHD9	9	1.11-1.20	0.93	MA	Unknown	[22]
TLE4						
CILP2	19	1.13	0.08	MA, CC	Unknown	[26,27]
DGKB	7	1.04-1.06	0.47-0.54	MA	Diacylglycerol kinase/Insulin action	[24,25]
DUSP9	X	1.09-1.27	0.12-0.77	MA	Phosphatase	[22,24]
FOLH1	11	1.10	0.09	GWAS	Transmembrane glycoprotein/Unknown	[24]
FTO	16	1.06-1.27	0.38-0.41	GWAS, MA	Metabolic regulator/Insulin action	[24,37]
GATAD2A	19	1.12	0.08	GWAS	Transcriptional repressor/Unknown	[24]
GCK	7	1.07	0.20	MA	Glucokinase/Insulin action	[25]
GCKR	2	1.06-1.09	0.59-0.62	MA	Glucokinase regulator/Insulin action	[24,25]
GIPR	19	1.10	0.27	GWAS	G-protein coupled receptor/Unknown	[24]
GRB14	2	1.07	0.60	MA, GCS	Adapter protein/Insulin action	[26,27]
HFE	6	1.12	0.29	MA	Membrane protein/Unknown	[38]
HHEX	10	1.12-1.13	0.53-0.60	AL, MA	Transcriptional repressor/	[22,24,34,39]
IDE					Intracellular insulin degradation/	
KIF11					Motor protein	
HMG20A	15	1.08	0.68	MA, GCS	Chromatin-associated protein/Unknown	[26,27]
HMGA1	6	1.34-15.8	0.10	GCS	Transcriptional regulator/Insulin action	[40-42]
HMGA2	12	1.10-1.20	0.09-0.10	MA	Transcriptional regulator	[22,24]
HNF1A	12	1.07-1.14	0.77-0.85	MA	Pancreatic and liver transcriptional activator	[22,24]
HNF1B	17	1.08-1.17	0.47-0.51	GCS, MA	Transcription factor/β-cell function	[22,24]
IGF2BP2	3	1.14	0.29-0.32	GWAS, MA	Binding protein/β-cell function	[22,24,34,35]
IRS1	2	1.09-1.12	0.64-0.67	GCS, MA	Insulin signaling element/Insulin action	[22,24,43]
JAZF1	7	1.10	0.52	MA	Zinc finger/β-cell function	[22,23]
KCNJ11	11	1.09-1.14	0.37-0.47	GCS, MA	Potassium channel/β-cell function	[22,24,34,44]
KCNQ1	11	1.08-1.23	0.44	GWAS	Potassium channel/β-cell function	[22,45,46]
KLF14	7	1.07-1.10	0.55	MA	Transcription factor/Insulin action	[22]
KLHDC5	12	1.10	0.80	MA, CC	Mitotic progression and cytokinesis/Unknown	[26,27]
LAMA1	18	1.13	0.38	GWAS	Cellular migration mediator/Unknown	[29]
MC4R	18	1.08	0.27	MA, CC	G-protein–coupled receptor/Unknown	[26,27]
MTNR1B	11	1.05-1.08	0.28-0.30	GWAS, MA	Melatonin receptor/β-cell function	[24,47-49]
NOTCH2	1	1.06-1.13	0.10-0.11	MA	Membrane receptor	[22-24]
PPARG	3	1.11-1.17	0.85-0.88	GCS, MA	Nuclear receptor/Insulin action	[22,24,34,50]
PRC1	15	1.07-1.10	0.22	MA	Cytokinesis regulator	[22]
PROX1	1	1.07	0.50	MA	Homeobox transcription factor/Insulin action	[25]
PTPRD	9	1.57	0.10	GWAS	Protein tyrosine phosphatase	[51]
RBMS1	2	1.11-1.08	0.79-0.83	MA	DNA modulator/Insulin action	[24,52]
SLC2A2	3	1.06	0.74	GWAS	Glucose sensor/β-cell function	[24]
SLC30A8	8	1.11-1.18	0.65-0.70	GWAS, MA	Zinc efflux transporter/β-cell function	[22,24,25,34,53]
SREBF1	17	1.07	0.38	GWAS	Lipid transcriptional regulator/Unknown	[24]
SRR	17	1.28	0.69	GWAS	Serine racemase	[51]
TCF7L2	10	1.31-1.71	0.26-0.30	LA, MA,GWAS	Participates in the Wnt signaling pathway/β-cell function	[21,22,24,34]
THADA	2	1.15	0.90	MA	Thyroid adenoma-associated protein/β-cell function	[22-24]
TH/INS	11	1.14	0.39	GWAS	Catecholamine synthesis/Unknown	[24]
TLE1	9	1.07	0.57	MA, CC	Transcriptional corepressor/Unknown	[26,27]
TP53INP1	8	1.06-1.11	0.48	MA	Proapoptotic protein/Unknown	[22]
TSPAN8	12	1.06-1.09	0.27-0.71	MA	Cell surface glycoprotein/β-cell function	[22-24]
LGR5					G-protein coupled receptor/β-cell function	
WFS1	4	1.10-1.13	0.60-0.73	GCS	Transmembrane protein/β-cell function	[22,24,54,55]
ZBED3	5	1.08-1.16	0.26	MA	Zinc finger/b-cell function	[22]

ZFAND6	15	1.01-1.11	0.60-0.72	MA	Zinc finger/β-cell function	[22,24]
ZMIZ1	10	1.08	0.52	MA, CC	Transcriptional regulator/Unknown	[26,27]
Haplogroup B	mtDNA	1.52	0.25	GCS		[56]
OriB	mtDNA	1.10	0.30	MA		[57]

Chr: Chromosome; MA: Meta-analysis; LA: Linkage analysis; GWAS: Genome-wide association study; GCS: Gene candidate study.

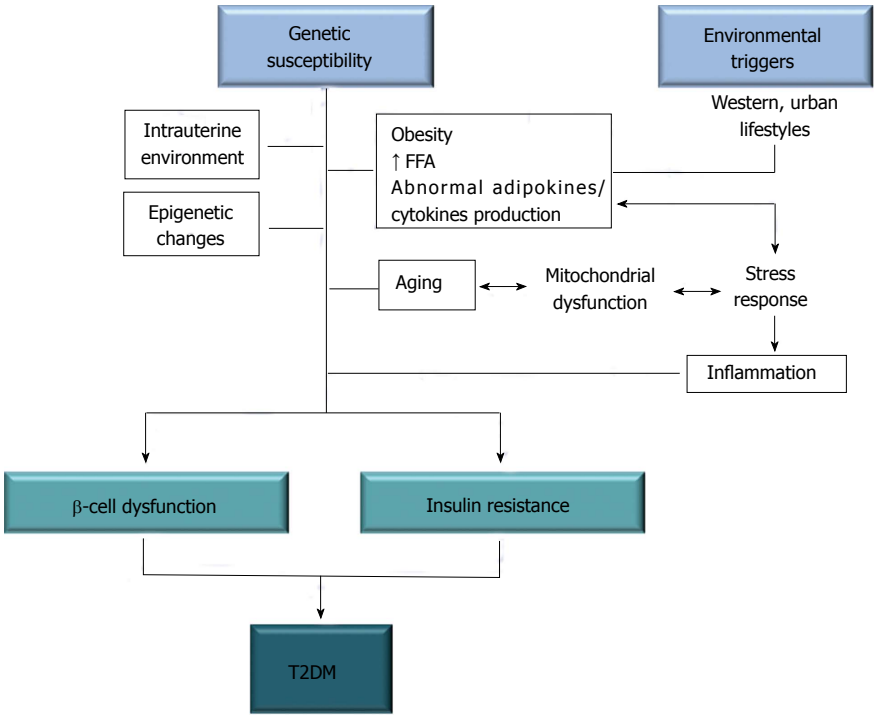

Figure 1 Overview of the pathogenic factors underlying development of type 2 diabetes mellitus. As a complex disease, T2DM is caused by a combination of genetic, environmental and lifestyle factors, all of which interact together to produce insulin resistance and β-cell dysfunction, leading to hyperglycemia, which is the clinical hallmark of diabetes. FFA: Free fatty acids. T2DM: Type 2 diabetes mellitus.

find links between genes and diseases across a substantial population. This strategy uses a database of over a million known genetic variants, which represent the majority of all common variants (minor allele frequency > 5%-10%), thus offering the possibility of simultaneously analyzing thousands of variations in a large number of patients and to perform meta-analysis of data from multiple studies. This methodology has helped to identify dozens of new associations between T2DM and genes with known or unknown functions (Table 1)[22-57], confirming some of the results from previous studies. However, despite the great potential of this approach, it is estimated that genetic variants identified through GWAS explain only 10% heritability for T2DM[58,59]. These relatively modest results can be explained taking into account some important limits of this strategy, such as the involvement of novel genetic variants not yet covered in the GWAS database, or the presence of variants with a frequency lower than the minimum threshold value. This means that the genes identified by GWAS so far are just the tip of the iceberg and that T2DM, far from being a condition limited to a few genetically and phenotypically prevalent forms, actually encompasses a heterogeneous group of genetically distinct disorders[18].

However, in many genetic studies carried out to date, the functional mechanism(s) by which the associated gene may increase susceptibility to T2DM is often poorly understood. In this respect, the intrinsic limitations of both the linkage analysis and GWAS are amplified by the fact that, in most cases, the genetic variants identified are located in non-coding regions of the DNA, whereby it becomes even more difficult to trace the role and influence of the associated gene in the development of the disease. In cases in which it was possible to ascertain the precise pathogenic mechanism, for example, through the study of association with the circulating levels of insulin or through the direct analysis of the gene's protein product, it has been seen that most of the genes identified are involved in pancreatic β-cell mass and/or function, thus with implications in insulin secretion defects (Table 1). This observation suggests that most of the risk associated with T2DM in the general population relates to genetic defects in β-cells, while peripheral insulin resistance predominantly suffers from the environmental component[18,19,60].

GENES INVOLVED IN β-CELL INSULIN SECRETION

Figure 2 depicts some of the genes whose alteration confers an elevated risk of T2DM. Using the analysis of functional or positional candidate genes, several variants have been identified, including polymorphisms of the gene insulin receptor substrate-1 (*IRS-1*)[22,24,43]. The Gly972Arg variant of IRS-1 determines a defect in the

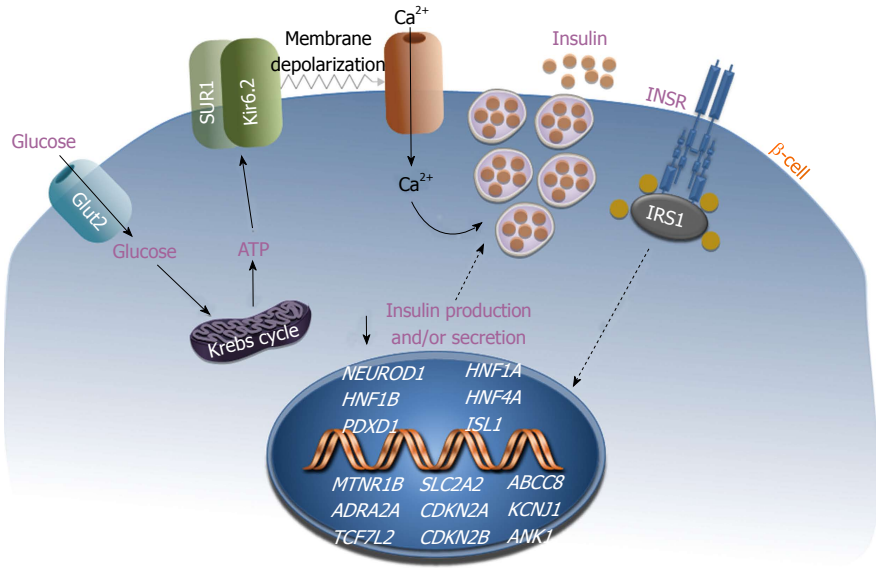

Figure 2 Schematic representation of the pancreatic β-cell. Reduced insulin secretion is shown in β-cells with gene variants linked to T2DM. Genes associated with defects in β-cell mass and/or function are indicated in white italic uppercase. T2DM: Type 2 diabetes mellitus.

binding of the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) which in pancreatic β-cells causes a marked decrease in insulin secretion in response to glucose and sulfonylureas[61]. Other polymorphisms implicated in T2DM have been identified in the *ABCC8* (also known as *SUR1*) and *KCNJ11* genes, whose protein products take place in the formation of the Adenosine triphosphate (ATP)-sensitive potassium channel/sulfonylurea receptor of the pancreatic β-cell. The therapeutic response to sulfonylureas is compromised in patients with mutations in these genes. Other genes whose mutations were initially considered responsible for the less common forms of diabetes mellitus have subsequently been associated with an increased risk of T2DM[19]. Among these are the hepatocyte nuclear factor-1 homeobox A (A*HNF1A*) gene, whose mutations are responsible for the most common monogenic form of MODY (MODY3), a form of maturity onset diabetes of the young (also known as HNF1A-MODY), and the gene hepatocyte nuclear factor-1 homeobox B (*HNF1B*), which determines a less frequent but more severe monogenic form of diabetes, the MODY5. Both of these genes encode nuclear transcription factors involved in the development and function of pancreatic islets.

As already mentioned, the association between *TCF7L2* gene polymorphisms and susceptibility to T2DM was highlighted initially by linkage studies and confirmed thereafter by GWAS. However, only recently has the role played by the transcription factor TCF7L2 in the β-cell insulin secretion become evident[62]. Another gene that has recently been associated with T2DM is the melatonin receptor 1B (*MTNR1B*) gene which encodes for the receptor of the pineal hormone melatonin, MTNR1B, that is involved in the regulation and facilitation of sleep. Genetic variants of the *MTNR1B* gene, associated with gain-of-function of the MTNR1B receptor protein and a reduction in insulin secretion, have been reported in diabetic patients with abnormalities in melatonin secretion and circadian rhythm disorders of the sleep-wake cycle[63]. Another example of genetic abnormality associated with β-cell dysfunction and the risk of T2DM involves the *ADRA2A* gene that encodes for the alpha 2A-adrenergic receptor, which mediates the adrenergic suppression of insulin secretion[60]. Diabetic patients with polymorphisms of the *ADRA2A* gene may have overexpression of the alpha 2A receptor, resulting in insulin secretion deficiency. In pancreatic islets obtained from diabetic patients carrying this variant, pharmacological treatment with alpha (2A)-AR antagonists rescued insulin secretion[64].

Recently, large scale GWAS meta-analyses and imputation-based GWAS studies have demonstrated that the ankyrin 1 gene, a gene encoding for a protein of the ankyrin family, is associated with T2DM in different ethnicities[26-28]. Ankyrin 1 is typically expressed in the erythrocytes and functions as an adaptor molecule between membrane and skeleton proteins. Interestingly, mutations of this gene are known to determine hereditary spherocytosis. How this protein can be implicated in T2DM is not yet understood; however, ankyrin 1 is also expressed in β-cells, where a cognate protein, ankyrin B, plays a role in regulating ATP sensitivity by interacting with the sulphonylurea receptor isoform SUR1.

Another recent study has identified new loci and variants in a large-scale gene-centric meta-analysis that included the *SLC2A2* (solute carrier 2A2) gene[24]. This gene encodes the glucose transporter Glut2, which is expressed in pancreatic β-cells, liver and kidney, and functions as a glucose sensor to maintain glucose homeostasis. These findings support a previously postulated role of Glut2 in T2DM[65]. Also, variants of genes involved in the cell cycle, like the *CDKN2A* and *CDKN2B* (cyclin-dependent kinase inhibitor 2A and 2B) genes, have been associated with T2DM. Although not proved in humans, data from animal models support the idea that these genetic variants may affect β-cell mass later in life[66].

GENES INVOLVED IN INSULIN RESISTANCE

The first step in the mechanism of action of insulin is

the interaction of the hormone with its specific receptor, the insulin receptor (INSR), on the cell surface of insulin responsive cells and tissues (Figure 3). The functional activation of INSR is a key moment in the pathophysiology of insulin action, followed by the selective activation of specific intracellular signaling pathways which are necessary for proper hormonal signal transduction. Although defects in INSR have been reported in a large number of patients with T2DM, mutations in the *INSR* gene have been found only in a small percentage (3%-4%) of these patients in whom genetic defects leading to receptor protein abnormalities were identified as cause of disease. However, certain patients with apparently normal *INSR* genes have reduced expression of both the INSR protein and INSR mRNA levels[13,18,19]. In these patients, it is possible that there are mutations in genes encoding transacting factors which regulate the level of *INSR* gene expression[40].

The mechanisms by which gene variants may impair insulin action in insulin target tissues are schematized in Figure 3. Among the genes involved in insulin resistance are those encoding for the glucokinase regulatory protein, GKRP, and the insulin-like growth factor- I , IGF- I . Genetic variants of these genes that predispose a person to develop insulin resistance have been recently identified by GWAS[25]. In addition, T2DM risk alleles at three loci (at *FTO*, *KLF14* and *PPARG*) have been associated with higher fasting insulin (which is consistent with a primary defect on insulin action) and reduced insulin sensitivity[22]. In particular, variations in the fat mass and obesity-associated (*FTO*) gene appear to influence predisposition to T2DM through a positive effect on body mass index and obesity. Instead, the Krüppel-like factor 14 (*KLF14*) gene is considered a super gene with the ability to control other genes linked to body fat. The risk alleles at *KLF14*, along with those at peroxisome proliferator-activated receptor gamma (*PPARG*), appear to have a primary effect on insulin action which, unlike the alleles at *FTO,* is not driven by obesity[22].

A recently uncovered gene implicated in T2DM is the growth factor receptor-bound 14 (*GRB14*) gene[26,27], which codes for the Grb14 adaptor protein. Grb14 contains a C-terminal SH2 domain implicated in the interaction with a number of tyrosine kinase receptors and signaling proteins, and a domain called BPS (between pleckstrin homology), also required for binding to the INSR. This protein has been shown to specifically attenuate insulin action by inhibiting the catalytic activity of the INSR in insulin target tissues[67]. Many other recently identified diabetes-associated genes play still unknown roles in the pathophysiology of T2DM. Among them, the sterol regulatory element-binding transcription factor 1 (*SREBF1*) gene, which is involved in the transcriptional regulation of lipid homeostasis[24], and the high mobility group 20A (*HMG20A*) gene, which encodes a chromatin-associated protein and has previously been associated with a greater incidence of diabetes in obese subjects[26,27].

THE HIGH MOBILITY GROUP A1 GENE

Among the group of genes recently associated with insulin resistance and T2DM is the *HMGA1* gene, which encodes the architectural transcription factor, High Mobility Group A1 (HMGA1), a nonhistone basic protein that binds to AT-rich sequences of DNA *via* AT hooks, facilitating the assembly and stability of a multicomponent enhancer complex, the "enhanceosome", which drives gene transcription[68]. We previously found that HMGA1 is a key regulator of *INSR* gene expression[69-71] (Figure 4). Consistent with these findings, we identified two patients with insulin-resistant T2DM who had defects in HMGA1 expression and concomitant decreased INSR mRNA and protein in muscle, fat and circulating monocytes[72]. These individuals had normal *INSR* genes but had a novel genetic variant (*c.*369del*) in the 3' noncoding region of the HMGA1 mRNA that contributed to the reduction of mRNA half-life and subsequent decline in HMGA1 expression. Epstein-Barr virus (EBV)-transformed lymphoblasts from these patients demonstrated defects in HMGA1 and INSR expression, indicating that the defects observed *in vivo* were not due to the altered metabolic state of the patients. In addition, the *in vitro* restoration of HMGA1 RNA and protein expression in these cells normalized *INSR* gene expression and restored both cell-surface INSR protein expression and insulin binding capacity[72]. The pathogenetic role of HMGA1 in T2DM was confirmed in genetically modified mice, in which the loss of HMGA1 expression (induced by disrupting the *HMGA1* gene) considerably decreased INSR expression in the major target tissues of insulin action[72], thus supporting the concept that functional *HMGA1* gene variants decrease INSR expression in human and mice.

In the context of these investigations, we later showed that four functional variants of the *HMGA1* gene, leading to reduced INSR expression, were associated with insulin resistance and T2DM[40]. The most frequent functional *HMGA1* variant, c.136-14_136-13insC (also designated rs146052672), was detected in 7%-8% of patients with diabetes in individuals of white European ancestry[40]. Analysis of cultured EBV-transformed lymphoblasts from patients with T2DM and the rs146052672 variant revealed that these cells had lower levels of HMGA1 and INSR protein than cells from either patients with wild-type T2DM or controls. Once again, in transformed lymphoblasts from the patients with the *HMGA1* rs146052672 variant, restoration of HMGA1 protein expression by complementary DNA transfection (in the sense but not antisense direction) restored INSR protein expression and insulin binding to these cells[40]. Although not replicated in a heterogeneous French population[73], the *HMGA1* rs146052672 variant was significantly associated with T2DM among Chinese[41] and Hispanic-American[38] individuals. Further evidence, implicating the HMGA1 locus as one conferring a high cross-race risk for the development of insulin

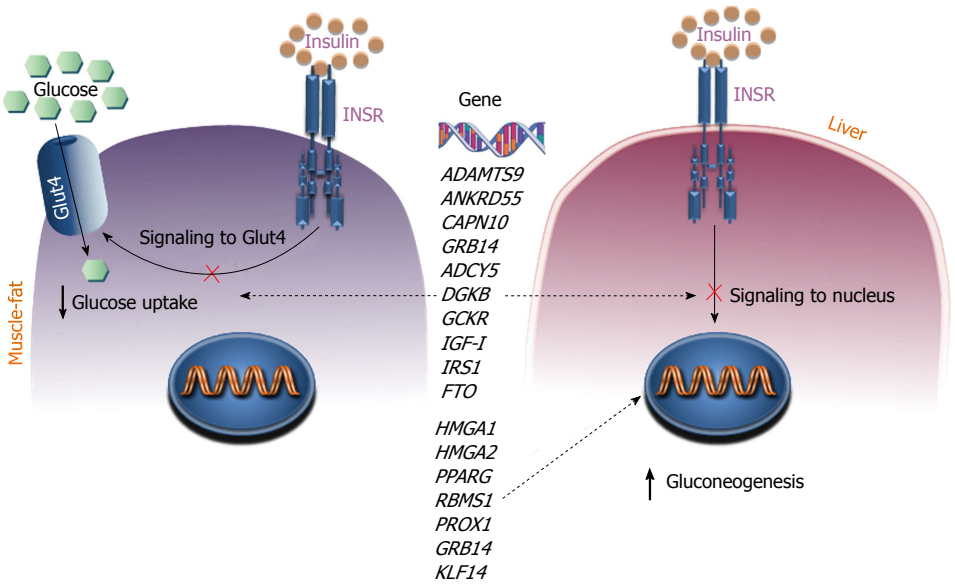

Figure 3 Mechanisms of insulin resistance. The figure shows the mechanisms by which gene variants may impair insulin action in the insulin target tissues muscle, fat and liver. Peripheral insulin resistance in muscle and fat reduces cellular glucose uptake, whereas insulin resistance in liver results in a failure to suppress glucose production and gluconeogenesis. Genes whose variations can influence the risk of developing insulin resistance and T2DM are indicated in black italic uppercase. T2DM: Type 2 diabetes mellitus.

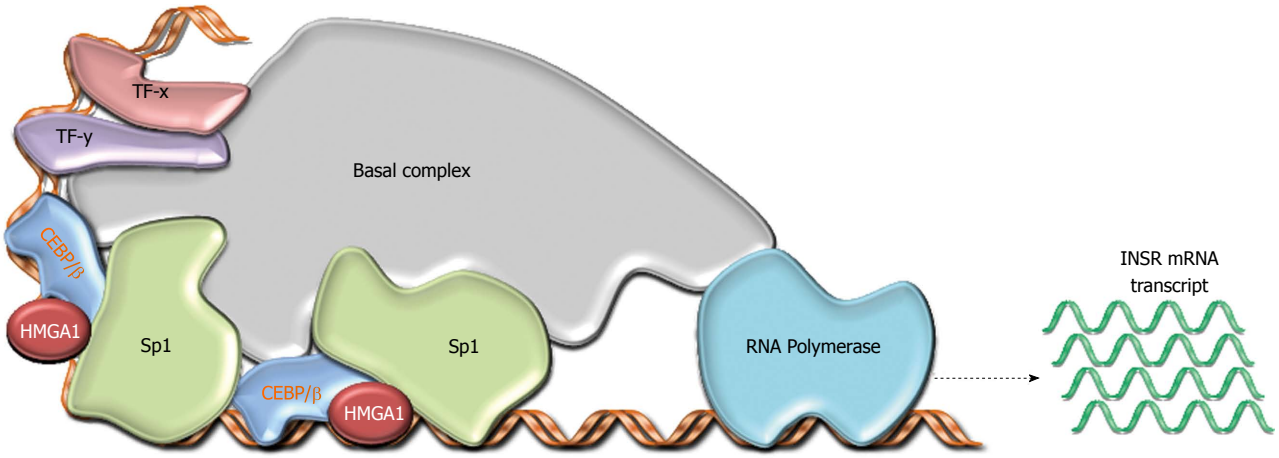

Figure 4 Model for the role of High Mobility Group A1 in type 2 diabetes mellitus. As a transcriptional regulator of the *INSR* gene, *HMGA1* gene variants may lead to decreased *INSR* gene transcription. This loss of insulin receptor (INSR) underlies the resultant insulin resistance and T2D in affected individuals. T2D: Type 2 diabetes.

resistant diseases, has been provided recently by showing that the *HMGA1* rs146052672 variant significantly associates with the metabolic syndrome in Italian and Turkish individuals and predisposes these (and other) populations to the unfavorable anthropometric and metabolic traits of the metabolic syndrome[74,42].

Overall, these data are consistent with the impression that the association of *HMGA1* gene variants with T2DM is accomplished through a pathogenetic mechanism related to peripheral insulin resistance. However, additional studies *in vitro* and *in vivo*, in normal and mutant mice, indicate that HMGA1, in addition to its role on *INSR* gene and protein expression, acts as a novel downstream target of the INSR signaling pathway[75], thus representing a critical nuclear mediator of insulin action and function. In this regard, evidence has been provided indicating that HMGA1 plays an essential role in the transcriptional regulation of a variety of insulin-target genes, such as the *IGFBP-1* gene, as well as the gluconeogenic genes *PEPCK* and *G6Pase*[76], contributing to the transcriptional regulation of glucose homeostasis.

PERSPECTIVES

Significant advances have been made in recent years in relation to the pathogenesis of T2DM. This has significantly improved our knowledge of one of the most serious health threats in the world, allowing identification of genes and pathways involved in the development and progression of the disease. It has recently become possible to acquire molecular and genetic level information from an individual (*i.e.*, DNA genotyping, gene expression, epigenomic profile, *etc.*). However, while such information is becoming increasingly available, how the identified genes and pathways impact on T2DM still remain largely unknown, due to the multifactorial nature of the disease. Understanding the pathogenesis of T2DM is necessary to enable the identification of prognostic and predictive biomarkers, as well as new therapeutic targets, which in turn should lead to improved outcomes in affected patients. Thus, once new therapeutic targets of

interest are identified, it is necessary to develop molecules that can rescue function to disease-associated genes or pathways and conduct studies that provide new strategies for the treatment of T2DM.

CONCLUSION

T2DM is a heterogeneous disease with a strong genetic component and familial inheritance. Considerable effort has been made in the last decades to identify genes that may explain all the diabetic phenotypes. Currently, however, genetic studies on T2DM can explain only a small percentage of its heritability. Until now, the *HMGA1* gene displays the strongest association with T2DM and its most frequent variant, rs146052672, confers the highest risk for human T2DM. Hence, from a strategic point of view, this finding suggests directing future research towards the identification of rare genetic variants with a stronger association, rather than common variants with a relatively small effect on the disease. It is evident that if a genetic variant confers a high susceptibility to T2DM it may become a useful biomarker to search for. For example, the genetic variants identified in the *HMGA1* gene may represent a predictive marker for early detection of T2DM, especially in those individuals with a family history of the disease. Moreover, variants in the human *HMGA1* gene may induce a different clinical course of disease compared to diabetic patients without the variant and may predict response to therapy, allowing identification of a priori patients who could most benefit from a specific pharmacological treatment[77]. Another important point in support of genetic studies in T2DM is the fact that they may integrate and improve our knowledge about the molecular mechanisms underpinning the pathophysiology of this disease.

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P- Reviewers: Hssan M, Panchu P, Rosa C **S- Editor**: Qi Y
L- Editor: Roemmele A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Origin and therapy for hypertriglyceridaemia in type 2 diabetes

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Received: November 5, 2013 Revised: March 8, 2014
Accepted: March 17, 2014
Published online: April 15, 2014

Abstract

Hypertriglyceridaemia (HTG) is a risk factor for cardiovascular disease (CVD) in type 2 diabetes and is caused by the interaction of genes and non-genetic factors, specifically poor glycaemic control and obesity. In spite of statin treatment, residual risk of CVD remains high in type 2 diabetes, and this may relate to HTG and atherogenic dyslipidemia. Treatment of HTG emphasises correcting secondary factors and adverse lifestyles, in particular, diet and exercise. Pharmacotherapy is also required in most type 2 diabetic patients. Statins are the first-line therapy to achieve recommended therapeutic targets of plasma low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol. Fibrates, ezetimibe and n-3 fatty acids are adjunctive treatment options for residual and persistent HTG. Evidence for the use of niacin has been challenged by non-significant CVD outcomes in two recent large clinical trials. Further investigation is required to clarify the use of incretin-based therapies for HTG in type 2 diabetes. Extreme HTG, with risk of pancreatitis, may require insulin infusion therapy or apheresis. New therapies targeting HTG in diabetes need to be tested in clinical endpoint trials. The purpose of this review is to examine the current evidence and provide practical guidance on the management of HTG in type 2 diabetes.

Key words: Diabetes; Triglyceride; Therapy

Core tip: Diabetic dyslipidemia relates collectively to hyperglycaemia, insulin resistance, hyperinsulinaemia, abdominal visceral adipose disposition, increased liver fat content, and dysregulated fatty acid metabolism. Insulin resistance in diabetes induces hypertriglyceridaemia by increasing the enterocytic production of chylomicrons and an impaired clearance capacity is also involved. Usual care for diabetic dyslipidemia is statin treatment, but a significant proportion of patients have residual dyslipidemia, related to hypertriglyceridaemia and atherogenic dyslipidemia. Current evidence supports the use of fenofibrate in type 2 diabetics with high triglyceride levels.

Original sources: Pang J, Chan DC, Watts GF. Origin and therapy for hypertriglyceridaemia in type 2 diabetes. *World J Diabetes* 2014; 5(2): 165-175 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i2/165.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i2.165

INTRODUCTION

Hypertriglyceridaemia (HTG) is an important risk factor for cardiovascular disease (CVD)[1] and is defined as a fasting plasma triglyceride concentration > 95th percentile for age and sex in a population. HTG may be as prevalent as 50% in type 2 diabetes and is often unresponsive to statin treatment[2,3]. We review recent evidence on the role of HTG in atherosclerotic CVD and provide practical guidance on the management of HTG in type 2 diabetes.

PATHOPHYSIOLOGY OF HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Triglycerides, which originate from the intestine postprandially or endogenously from the liver, are packaged into lipoprotein particles containing apolipoprotein B-48 (apoB-48; chylomicrons) and apolipoprotein B-100 (apoB-100; very-low density lipoprotein, VLDL), respectively. Abnormalities in triglyceride-rich lipoprotein (TRL) metabolism are cardinal features of type 2 diabetes. Metabolic dysregulation resulting in HTG include enhanced hepatic secretion of TRL due to insulin resistance and delayed clearance of TRL involving lipoprotein lipase (LPL)-mediated lipolysis. Several genes causing loss of function of LPL can result in severe HTG, such as *LPL*, *APOC2*, *APOA5*, *GPD1*, *CPIHBP1* and *LMF1*[4,5]. Very few patients will have a monogenic disorder. Individuals with severe HTG are likely to be homozygous or compound heterozygous for mutations which impair the TRL catabolic pathway. However, HTG in type 2 diabetes due to several genes with mild effects that interact with non-genetic factors is probably more likely. These non-genetic factors include hyperglycaemia, alcohol abuse, concomitant medication, sedentary lifestyle, chronic kidney disease and insulin resistance[6].

Insulin resistance activates *de novo* lipogenesis, resulting in oversecretion of hepatic TRLs. This is also evident in the postprandial state, with enterocytic oversecretion of TRLs in the form of chylomicrons. With both secretion pathways on overdrive, competition between the TRLs and their remnants for lipolytic and receptor-mediated clearance further induces HTG. Insulin resistance is also associated with increased rates of apolipoprotein C-Ⅲ (apoC-Ⅲ) secretion, which further impairs receptor-mediated uptake of hepatic chylomicron remnants[7]. Glucose has also found to activate apoC-Ⅲ transcription, which may be the link between hyperglycaemia, HTG and CVD in type 2 diabetics[8].

Both LPL and hepatic lipase (HL) control the clearance of triglycerides. HL plays a particularly important role in the delipidation cascade from VLDL to LDL. Triglyceride-rich VLDL derives small, dense LDL particles which are more susceptible to oxidation[9]. Additionally, increased TRL in postprandial diabetic dyslipidemia leads to the exchange of TRL-triglyceride for HDL-cholesteryl ester and hence, triglyceride enrichment of HDL *via* cholesteryl ester transfer protein (CETP). CETP progressively decreases postprandially and limits the efficient removal of cholesterol[10]. Triglycerides in HDL are good substrates for hepatic lipase which leads to the production of small dense HDL particles and enhanced apolipoprotein A-Ⅰ (apoA-Ⅰ) clearance[11].

Given that HTG is related to a plethora of risk factors, the lack of independent association between triglyceride and CVD is expected[12], although two recent Mendelian randomisation studies have shown a causal association between variations in two related genes (*LPL* and *APOA5*) and myocardial infarction[13]. This supports that TRL causes CVD, and this probably applies to diabetes.

Hence, diabetic dyslipidemia relates collectively to hyperglycaemia, insulin resistance, hyperinsulinaemia, abdominal visceral adipose disposition, increased liver fat content, and dysregulated fatty acid metabolism. Diabetic dyslipidemia may also be exacerbated by chronic kidney disease and by co-prescribed medications, such as thiazide diuretics, non-selective beta-blockers and steroids.

MANAGEMENT OF HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Measurement and assessment

Triglyceride concentration is commonly measured with a fasting lipid profile. The fasting triglyceride level facilitates the calculation of the LDL cholesterol by the Friedewald equation[14]. Non-fasting triglyceride concentrations are reflective of the postprandial state and can be useful as a simple and practical screening test for HTG. A second non-fasting measurement is recommended if the initial triglyceride is > 2.0 mmol/L. Two or more measurements of elevated triglyceride in both postabsorptive and postprandial states are clinically indicative of HTG. Categories of HTG are differentially defined in international guidelines (Table 1).

Non-HDL cholesterol is another appealing method of assessment as it does not attract additional costs. Non-HDL cholesterol (total cholesterol minus HDL-cholesterol) does not rely on a fasting triglyceride concentration and provides a simple amalgamated measure all the atherogenic lipoproteins[15]. ApoB, on the other hand, does not adequately reflect chylomicron remnants and involves additional laboratory expenses. Discordance between non-HDL cholesterol and apoB measures, particularly in patients with type 2 diabetes and HTG, questions its value in assessing risk and defining treatment targets[16]. In the context of statin-treated patients, a meta-analysis has shown that non-HDL cholesterol is superior in its association with risk of future major cardiovascular events compared with LDL cholesterol and apoB[17]. Other TRL markers such as remnant-like particle cholesterol, apoC-Ⅲ and apoB-48 are expensive and are yet to be clinically established.

The hypertriglyceridaemic waist (HTWC) phenotype has suggested to be useful in assessing glucometabolic risk[18-21], in particular, among patients with a family history of diabetes[22]. The HTWC phenotype is defined by a waist circumference of ⩾ 90 cm in men and ⩾ 85 cm in women and triglyceride concentration ⩾ 2.0 mmol/L. Men with the HTWC phenotype have been shown to have a four-fold risk of diabetes compared to those with waist circumference and triglyceride in the normal ranges[23]. There is also a two-fold risk for development of coronary artery disease (CAD) in women[24] and an overall deterioration of cardiometabolic risk[25] in relation to progression of type 2 diabetes[26].

Table 1 Clinical categorisation of hypertriglyceridaemia according to guidelines based on fasting triglyceride concentrations

Ref.	Year published	Triglyceride categories	Triglyceride concentration (mmol/L)
National institutes of Health[31]	2001	Normal	1.7
		Borderline high	1.7-2.3
		High	2.3-5.6
		Very high	> 5.6
Rydén *et al*[33]	2011	Desirable	< 1.7
		Elevated	1.7-5.5
		Very high	5.5-25.0
		Extremely high	> 25.0
Berglund *et al*[34]	2012	Normal	< 1.7
		Mild	1.7-2.3
		Moderately high	2.3-11.2
		Severely high	11.2-22.4
		Very severely high	> 22.4
Hegele *et al*[37]	2013	Normal	< 2.0
		Mild-to-moderate	2.0-10.0
		Severe	> 10.0

Table 2 Clinical guide for the assessment and treatment of hypertriglyceridaemia in type 2 diabetes

Steps	
1	Obtain fasting lipid profile
2	Classify LDL-cholesterol concentration (primary target of therapy) < 2.60 mmol/L – optimal 2.60-3.39 mmol/L – above optimal 3.40-4.14 mmol/L – borderline high 4.15-4.90 mmol/L – high > 4.90 mmol/L – very high Establish therapy: LDL-cholesterol > 2.60 mmol/L – initiate dietary and lifestyle modifications LDL-cholesterol > 3.40 mmol/L – consider pharmacotherapy simultaneously with dietary and lifestyle modifications
3	Identify presence of atherosclerotic disease Clinical coronary heart disease Symptomatic carotid artery disease Peripheral artery disease
4	Assess: Glycaemic control Obesity Dietary intake (*e.g.*, Fructose, simple sugars, caloric intake) Physical activity Determine presence of other risk factors: Smoking Hypertension Family history of premature coronary heart disease (*i.e,*. in first-degree relative, male < 55 years, female < 65 years) Low HDL-cholesterol, < 1.0 mmol/L
5	Order of treatment considerations: Improve glycaemia (dietary and lifestyle modifications) Treat secondary risk factors Statins Fibrates n-3 fatty acids/niacin
6	Treat elevated triglyceride if triglyceride concentrations are > 2.30 mmol/L after LDL-cholesterol concentration target of < 2.60 mmol/L is reached Target non-HDL cholesterol (< 3.40 mmol/L) Triglyceride > 2.30 mmol/L – intensify LDL-lowering therapy or add fibrate Triglyceride > 5.60 mmol/L – very low-fat diet (< 15% of calories from fat), weight management, physical activity and add fibrate

Adapted from the NCEP ATP Ⅲ guidelines[31]. LDL: Low density lipoprotein; HDL: High density lipoprotein.

Guidelines and recommendations

Guidelines for managing HTG in diabetes have been published, with lifestyle modifications being first-line therapy followed by statins, fibrates, n-3 fatty acids and/or niacin[27-30]. The national cholesterol education program (NCEP) adult treatment panel (ATP) Ⅲ guidelines recommend LDL cholesterol as the primary treatment target and non-HDL cholesterol as a secondary target, with the exception of a fasting triglyceride > 5.60 mmol/L, only then, triglyceride becomes the primary target owing to the risk of pancreatitis[31]. A simplification of the NCEP ATP Ⅲ guideline is presented in Table 2. Regardless of atherosclerotic disease and presence of other cardiovascular risk factors, type 2 diabetes is considered a coronary heart disease risk equivalent by the NCEP ATP Ⅲ.

The American Diabetes Association (ADA)/American College of Cardiology Foundation consensus statement recommends a non-HDL cholesterol target of 3.40 mmol/L in diabetic patients with no other cardiovascular risk factor and a target of 2.60 mmol/L if there is one or more cardiovascular risk factor such as hypertension, smoking, dyslipidemia and family history of CAD[32]. The LDL cholesterol target is 2.60 and 1.80 mmol/L, respectively[32] or alternatively a 30%-40% reduction from baseline levels[30]. The ADA position statement is the only guideline that provides desirable targets for triglyceride levels for patients with type 2 diabetes: less than 1.70 mmol/L[30]. Both the NCEP ATP and ADA guidelines place emphasis on weight loss and physical activity. A summary of recommended treatment targets is presented in Table 3.

The Scientific Statement from the American Heart Association (AHA) on triglycerides and CVD particularly emphasises the dietary and lifestyle modifications (weight loss, macronutrient distribution and aerobic exercise) for the treatment of elevated triglycerides, presenting a practical algorithm for screening and management[28]. The European Society of Cardiology (ESC) guidelines on diabetes and CVD developed in collaboration with the European Association for the Study of Diabetes (EASD) suggests targeting residual risk in patients with elevated TG (> 2.2 mmol/L), with dietary and lifestyle advice and improved glucose control[33], post first-line treatment. The Endocrine Society task force agrees with the NCEP ATP Ⅲ treatment goals and recommends fibrates as first-line treatment for lowering triglycerides in patients at-risk for pancreatitis[34].

The International Atherosclerosis Society position paper recognises the atherogenicity of VLDL and triglycerides and also favours non-HDL cholesterol as the main target for therapy, optimally at < 3.40 mmol/L[35]. The American College of Cardiology (ACC)/AHA published a new clinical practice guideline for the treatment of elevated blood cholesterol in people at high risk for CVD.

Table 3 Recommended treatment targets for diabetic dyslipidaemia

		NCEP ATP Ⅲ[31]	ADA[30]	NVDPA[128]	European Guidelines[33]
LDL-cholesterol (mmol/L)	Very high risk	< 1.8	< 1.8	< 2.0	< 1.8
	High risk	< 2.6	< 2.6	< 2.0	< 2.5
Triglycerides (mmol/L)			< 1.7	< 2.0	< 1.7
HDL-cholesterol (mmol/L)	Male		> 1.0	⩾ 1.0	> 1.0
	Female		> 1.3	⩾ 1.0	> 1.2
Non-HDL cholesterol (mmol/L)	Very high risk	< 2.6	< 2.6	< 2.5	< 2.6
	High risk	< 3.4	< 3.4	< 2.5	< 3.3
ApoB (g/L)	Very high risk		< 0.8		< 0.8
	High risk		< 0.9		< 1.0

NCEP ATP Ⅲ: Third Report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult treatment panel Ⅲ); ADA: American diabetes association; NVDPA: National vascular disease prevention alliance of australia; LDL: Low density lipoprotein; HDL: High density lipoprotein.

The guidelines do not provide recommendations for specific LDL-cholesterol or non-HDL targets and instead defines four major groups of primary and secondary prevention patients for whom LDL lowering is proven to be most beneficial[36]. Future guidelines to cover the treatment of HTG are proposed. A recent review by Hegele *et al*[37] recommended the simplification and redefinition of HTG: < 2.0 mmol/L as normal, 2.0-10.0 mmol/L as mild-to-moderate and > 10.0 mmol/L as severe; with desirable targets of < 1.7 mmol/L for triglycerides, < 2.6 mmol/L for non-HDL cholesterol and < 0.8 g/L for apoB in high-risk patients

Treatment of HTG depends on its severity, co-existing lipid abnormalities and overall cardiovascular risk. Severe HTG serves as increased risk of pancreatitis and warrants treatment to acutely reduce triglyceride levels. Current therapeutic strategies include diet and lifestyle modification, pharmacotherapy and in rare cases, continuous insulin infusion and apheresis.

Dietary and lifestyle modifications

Lifestyle interventions are central for controlling hyperglycaemia and HTG in patients with type 2 diabetic patients and impaired fasting glucose. These interventions include weight reduction, altered dietary composition, exercise and regulation of alcohol consumption. In type 2 diabetes, modest (5%-10%) weight loss can lower plasma triglyceride levels by up to 25%[38,39] and normalise postprandial triglyceride concentration[40]. Physical activity can aid the maintenance of weight loss achieved through caloric restrictions[41], although evidence for linking lifestyle modifications and sustained weight is limited[42].

The recently published look AHEAD trial, an intensive lifestyle intervention in type 2 diabetics, employing weight loss through caloric restriction and increased physical activity did not reduce the rate of cardiovascular events[43]. Whether alterations in dietary composition, such as with the Mediterranean diet, improves clinical outcome in diabetes warrants additional investigation[44], though the Mediterranean and low-carbohydrate diet can produce a greater reduction in triglyceride levels compared to the restricted-calorie diet in moderately obese individuals[45,46]. Plant sterols have been suggested for lowering TG in individuals with overt HTG[47]. Alcohol abstinence in patients with excessive alcohol intake can markedly lower plasma triglyceride levels[48,49]. Smoking cessation is also imperative[50].

Pharmacotherapy

Statin monotherapy: Statin therapy is the cornerstone of treatment of dyslipidemia in diabetes. Whilst reaching the LDL cholesterol target in most patients, only modest effects are exerted on triglyceride and HDL cholesterol. Hence, diabetics with HTG often have residual CVD risk[51] in spite of an optimal LDL cholesterol target. Statins may lower plasma triglyceride by increasing lipolysis and the clearance of TRLs, particularly with potent statins such as atorvastatin and rosuvastatin (up to 26% and 28% reduction in plasma triglyceride, respectively)[52-54]. Large statin outcome trials have supported its use in reducing coronary events and mortality[55-58]. All trials to-date have not specifically selected for HTG and in diabetics. However, sub-group analyses have been undertaken showing risk prevention with pravastatin[59], simvastatin[60] and rosuvastatin[61] in a subset of patients with high plasma triglyceride, recently reviewed by Maki *et al*[62], and supporting statins as first line of therapy. Whilst use of higher doses of statin has been linked to incidence of diabetes[63-65], the benefits of statin therapy for reducing CVD risk and events are outweighed for all diabetic patients with high CVD risk[57,63]. Aminotransferase, creatine kinase, creatinine and glucose should be monitored prior to initiating statins and before initiating a second agent, if required.

Fibrates and statin-fibrate combination: Fibrates (gemfibrozil, fenofibrate) act on peroxisome proliferator-activated receptor alpha. Fibrates decreases hepatic VLDL secretion and can confer an up to 30% reduction in plasma triglyceride, TRL remnants and apoB[66]. Five fibrate trials have undertaken secondary analyses in high triglyceride subgroups[67-79], two of these trials were in type 2 diabetic patients[70-72] and one had a subset of diabetics[73,74]. Collectively, these trials advocate the use of fibrates in reducing CVD events among patients with

a high triglyceride and low HDL cholesterol levels[75-78]. Of note, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study showed that fenofibrate decreased progression of diabetic retinopathy[79], though unrelated to dyslipidemia, and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study also showed a delay in the onset of eye complications[80]. Meta-analyses suggest that fibrates are useful for treatment of HTG[76] in diabetic patients[71,81,82]. Every 0.10 mmol/L reduction in triglyceride with fibrates confers a 5% reduction in CVD event, although no benefits were found on cardiovascular mortality[77,78].

Niacin and statin-niacin combination: Niacin can decrease plasma triglyceride by 30%[83] *via* the inhibition of hepatic diacylglycerol acyltransferase-2 (DGAT-2), a rate-limiting enzyme of triglyceride synthesis. Despite the earlier studies showing reduced mortality[84] and regression of subclinical atherosclerosis[85-87], the current use of niacin has been challenged by two large recent clinical trials which have failed to show significant benefits on CVD events[88,89] in spite of positive changes in lipid parameters. Both trials have limitations. The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health (AIM-HIGH) study was underpowered and confounded by the higher statin and/or ezetimibe doses to match LDL cholesterol between groups[88]. The Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study is the largest extend-release niacin trial to-date combined with laropiprant, a prostaglandin D2 inhibitor[89]. Despite no significant benefit on primary CVD endpoints, a recent sub-analysis in patients with both high triglyceride (> 2.24 mmol/L) and low HDL cholesterol (< 0.85 mmol/L) showed a trend towards benefit with niacin, although not reaching statistical significance (HR = 0.74, $P = 0.073$)[90]. Of note, the lack of potential benefit or harm in the HPS2-THRIVE study may not necessarily be due to niacin, but potentially to laropiprant. The safety of niacin use in type 2 diabetes has previously been questioned owing to impairment in glycaemic control and insulin sensitivity[91-93]. However, two prospective trials have showed that the effect of niacin on glycaemic control is minimal in a majority of patients with stable diabetes[94] and with no changes in low-dose (1 g/d) niacin[95].

Ezetimibe and statin-ezetimibe combination: Ezetimibe inhibits intestinal cholesterol absorption and primarily lowers LDL cholesterol *via* the Niemann-Pick C1-Like 1 protein. Ezetimibe has minimal effects in lowering plasma fasting triglyceride (8%)[96]. A more prominent effect is observed in ameliorating postprandial lipaemia and lowering TRL remnants in spite of background statin[97,98]. In a 6-wk trial of simvastatin-ezetimibe vs. simvastatin monotherapy, fasting and postprandial plasma triglyceride and apoB-48 concentrations were lowered in type 2 diabetic patients[99]. However, intensive lipid lowering with a statin plus ezetimibe may not consistently lower subclinical carotid atherosclerosis in type 2 diabetes, although progression of carotid artery intima-media thickness was inhibited with the combination[100,101]. The Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) study that is currently entering completion will endeavour to provide definitive evidence for the role of ezetimibe in high risk subjects on optimal statin therapy[102,103].

n-3 fatty acid and statin-n-3 fatty acid combination: Supplemental n-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are well known to improve HTG[104]. However, recent clinical outcome trials with have failed to show significant CVD benefits in high risk subjects including diabetics[105,106]. Both trials were undertaken against a background of optimal therapy, including statins. Also, patients were not selected for elevated plasma triglyceride levels. Pure EPA (1800 mg/d), added to statin therapy, showed promise in the Japan Eicosapentaenoic acid Lipid Intervention Study (JELIS) with major coronary events reduced by 19% ($P = 0.011$) in hypercholesterolaemic patients[107]. Two 12-wk EPA (AMR101) intervention trials in patients with very high[108] and persistent[109] baseline triglyceride observed significant reductions in triglyceride levels. The greatest decrease was seen in the highest triglyceride tertile where there was a 31% reduction compared to placebo on 4 g/d of AMR101. The Reduction of Cardiovascular Events with EPA-Intervention Trial (REDUCE-IT) is in progress and will endeavour obtain the CVD outcome data with AMR101 4 g/d in high-risk patients with HTG and at-target LDL cholesterol on statin therapy[110]. There are also recent data suggesting an increased risk of prostate cancer with high dietary intake of n-3 PUFAs[111]. Hence, caution is warranted when recommending long-term intake.

Incretin-based therapy: Incretins, such as glucagon-like peptide-1 (GLP-1), are insulinotropic, gut-derived hormones secreted in response to diet. GLP-1 receptor analogs such as liraglutide and exenatide, delay gastric emptying and this parallels the reduction in postprandial triglyceride response[112]. This mechanism may ameliorate impaired TRL metabolism in type 2 diabetes. By increasing plasma concentrations of GLP-1, Dipeptidyl peptidase-4 (DPP-4) inhibitors, such as sitagliptin, saxagliptin and alogliptin, can improve insulin sensitivity, β-cell function[113] and postprandial glycaemia[114] and lipaemia[115]. These agents could potentially prevent CVD events, independent of changes in glucose and lipid metabolism. A recent saxagliptin outcome trial failed to demonstrate significant changes in ischaemic events, though the rate of heart failure increased significantly[116]. Similarly, a trial in type 2 diabetic patients post-acute coronary syndrome with aloglipitin did not improve cardiovascular event rates compared with placebo[117]. Further investigation is required to clarify

their mechanism and use in type 2 diabetes.

MANAGEMENT OF SEVERE HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES

Insulin infusion, apheresis and gene replacement therapy

In severe cases of diabetic HTG and poorly controlled diabetes, continuous intravenous insulin infusion appears to be beneficial in restoring serum glucose and triglyceride[118]. Most of these patients will have an underlying genetic defect in TRL metabolism. A recent study in a group of 15 diabetics with a median triglyceride concentration of 26.23 mmol/L at admission had their triglyceride levels corrected to a median of 5.75 mmol/L at discharge with an average of 48 h of continuous insulin infusion[119]. For prevention of recurrent severe HTG in susceptible patients, counselling on medication adherence and long-term diet and lifestyle medications should be considered[120].

In extremely severe HTG and drug refractory HTG, plasma apheresis may be required[121,122], particularly with severe chylomicronaemia complicated by acute pancreatitis. A single session of apheresis can dramatically lower excessive triglyceride levels, 65.8% reduction in 2 h[123,124]. This method of triglyceride lowering is only indicated in medical emergencies owing to high costs and limited availability[125]. Further study is required to clarify the role of plasma exchange in the treatment of hyperlipidaemic pancreatitis.

In patients genetically diagnosed with familial LPL deficiency, Glybera® (alipogene tiparvovec; Amsterdam Molecular Therapeutics, Amsterdam, the Netherlands) is the first approved gene-replacement therapy[126,127]. Glybera® has only been studied in 27 patients, in whom the agent was well tolerated and with plasma triglyceride concentration significantly lowered with reduced rates of acute pancreatitis[126]. Long-term follow-up data and cost-effectiveness studies is warranted[126,127].

CONCLUSION

HTG is common in type 2 diabetes. HTG associates with a spectrum of cardiometabolic risk factors and increases CVD risk in type 2 diabetes. Dietary and lifestyle modification involving weight loss and exercise is fundamental to the management of HTG. Improved glycaemic control with use of metformin, DPP-4 inhibitors and insulin can also improve HTG. The expression of HTG in context of diabetes may depend on co-existing monogenic and/or multigenic disorders of lipid metabolism, such as familial combined hyperlipidaemia, familial hypertriglyceridaemia and type Ⅱ hyperlipoproteinaemia. Statins are the first-line of lipid-lowering therapy to target LDL cholesterol and triglycerides. Current evidence supports the use of fenofibrate in type 2 diabetics, with high triglyceride and low HDL, but also to prevent and treat diabetic retinopathy. More evidence is required from CVD outcome trials for the other add-on options, some of which are currently underway. Several new therapies with potential applications for treating HTG are DGAT inhibitors, microsomal triglyceride transfer protein inhibitors, and apoC-Ⅲ antisense oligonucleotides. These will agents will require to be tested for efficacy, safety and cost-effectiveness in future clinical trials.

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P- Reviewers: Cheung BMY, Ramana KV **S- Editor**: Zhai HH
L- Editor: A **E- Editor**: Wu HL

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Functional foods-based diet as a novel dietary approach for management of type 2 diabetes and its complications: A review

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Received: November 24, 2013 Revised: January 11, 2014
Accepted: April 11, 2014
Published online: June 15, 2014

Abstract

Type 2 diabetes is a complicated metabolic disorder with both short- and long-term undesirable complications. In recent years, there has been growing evidence that functional foods and their bioactive compounds, due to their biological properties, may be used as complementary treatment for type 2 diabetes mellitus. In this review, we have highlighted various functional foods as missing part of medical nutrition therapy in diabetic patients. Several *in vitro*, animal models and some human studies, have demonstrated that functional foods and nutraceuticals may improve postprandial hyperglycemia and adipose tissue metabolism modulate carbohydrate and lipid metabolism. Functional foods may also improve dyslipidemia and insulin resistance, and attenuate oxidative stress and inflammatory processes and subsequently could prevent the development of long-term diabetes complications including cardiovascular disease, neuropathy, nephropathy and retinopathy. In conclusion available data indicate that a functional foods-based diet may be a novel and comprehensive dietary approach for management of type 2 diabetes.

Key words: Type 2 diabetes; Insulin resistance; Functional foods; Whole grain; Legumes; Nuts; Fruits; Herbs or spices; Vegetables; Prebiotics; Probiotics

Core tip: Medical nutrition therapy (MNT) is a main part of type 2 diabetes management. Apparently the therapeutic and medicinal properties of foods maybe a missing step during MNT process, and could enhance the effectiveness of dietary management of type 2 diabetes.

Original sources: Mirmiran P, Bahadoran Z, Azizi F. Functional foods-based diet as a novel dietary approach for management of type 2 diabetes and its complications: A review. *World J Diabetes* 2014; 5(3): 267-281 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/267.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.267

INTRODUCTION

Type 2 diabetes is a metabolic disorder characterized by hyperglycemia, developing insulin resistance, β-cell dysfunction and impaired insulin secretion[1,2]. Multiple metabolic disorders including impaired lipid and lipoprotein metabolism, oxidative stress (over production of free

radicals and defect in endogenous antioxidant defense system), sub-clinical inflammation, vascular endothelial dysfunction and hypertension are commonly accompanied by type 2 diabetes[3-5]; these metabolic disorders lead to long-term pathogenic conditions such as micro- and macro-vascular complications including neuropathy, retinopathy, nephropathy, and a decreased quality of life and an increased mortality rate[6,7].

Despite availability of many pharmacological interventions including oral hypoglycemic agents and insulin therapy for diabetes management, current evidence shows an alarming rising trend in the occurrence of undesirable complications among these patients[1].

Medical nutrition therapy (MNT) is also a main part of type 2 diabetes management; estimation of energy and nutrients requirements, carbohydrate counting as well as glycemic index and glycemic load, recommendation for dietary fats and cholesterol and protein intakes, explanation the foods exchange list for patients and common important recommendations for a healthy diet are the main components of diet planning in type 2 diabetic patients[8,9]; however it is not clear whether this approach *per se* is sufficiently adequate for prevention of long-term complications of diabetes. Administration of various supplements, including antioxidant vitamins, fibers, ω_3 fatty acids, numerous nutraceuticals, and herbs has also been proposed for glycemic control but data available supporting these recommendations for diabetic patients are insufficient[10-14]. Apparently the therapeutic and medicinal properties of foods maybe a missing step during MNT process, and could enhance the effectiveness of dietary management of type 2 diabetes.

During the past two decades, the concept of functional food is fast expanding; functional foods beyond the basic nutritional functions have potential benefits to promote health and reduce the risk of chronic diseases and have hence been given much attention[15,16]. In recent years, researchers have focused on properties of the bioactive compounds of functional foods in the control of various aspects of diabetes mellitus; some protective effects of these compounds and food sources have been investigated *in vitro* and *in vivo*, and several clinical trials have even confirmed these advantages in diabetic patients[17-19].

Here, based on the multiple biological properties of functional foods and their bioactive compounds, a functional foods-based diet has been hypothesized as a novel and comprehensive dietary approach for management of type 2 diabetes and prevention of long-term complications.

RESEARCH

The evidence cited in this review was obtained through searches in PubMed, Scopus, and Google scholar using the following key words: "Type 2 diabetes or hyperglycemia", "insulin resistance", "cardiovascular disease", "obesity", "metabolic syndrome", "oxidative stress", "inflammation", long-term diabetic complications" in combination with "functional foods", "nutraceuticals", "bioactive food compounds", "fiber", "polyphenols", "whole grain", "legumes", "nuts", "fruits", "herbs or spices" "vegetables", "prebiotics", "probiotics", and "bioactive peptides". Relevant articles of acceptable quality were used. Briefly, in this article we tried to highlight some of the following important functional foods including whole grains, phytochemical-rich fruits and vegetables, legumes, nuts, dairy products, green tea and some spices, as required components of a health-promoting diet for diabetic patients.

Whole grains

Grains and cereal-based products are the basic sources providing energy and carbohydrate in human diets. Since the dietary carbohydrate sources in type 2 diabetic patients play a determining role in glycemic and insulin secretary response, the use of functional grains including whole grain cereals, and bakery products prepared using whole wheat, rye, oat, and barley is the first step in planning of a functional foods-based diet.

Some previous studies report that dietary carbohydrate modification in patients with metabolic syndrome resulted in favorable metabolic consequences especially increased insulin sensitivity, decreased adipocyte cell size, and modulated expression of adipose tissue genes involved in insulin signaling pathways (insulin-like-growth-factor binding protein-5, insulin receptors, hormone-sensitive lipase[20,21].

Compared to refined grains, whole grains (WGs) have more non-digestible complex polysaccharides including soluble and insoluble fibers, inulin, β-glucan, and resistant starches, as well as non-carbohydrate functional components including carotenoids, phytates and phytoesterogens, phenolic acids (ferulic acid, vanilic acid, caffeic acid, syringic acid, *P*-cumaric acid), and tocopherols. The most well-known protective effects of whole grain-based products against obesity, type 2 diabetes, cardiovascular diseases, hypertension, metabolic syndrome and various types of cancer, have been attributed to these bioactive compounds[22-25]. Among the several mechanisms available in current data regarding the beneficial effects of WGs and cereal-based products in diabetic patients, some of the more important are that bioactive compounds of WGs could effectively regulate glycemic response, increase insulin sensitivity, improve pancreatic β-cell functions and increase insulin secretion[26,27]. High contents of inulin and β-glucan, main soluble and fermentable fibers in WGs, in addition to their hypolipidemic and hypoglycemic effects, act as prebiotics in the gut and modulate gut microbiota *via* stimulation of growth and activity of bifidobacteria and lactic acid bacteria[28,29], effects leading to more metabolic responses (Figure 1).

Long-term follow-ups of diabetic patients indicate that higher consumption of whole grain, cereal fiber, bran, and germ were associated with decreased all-cause and cardiovascular disease-cause mortality[30]. Epidemiological studies also confirmed that regular consumption of WGs products could modify the main risk factors of atherosclerotic diseases including triglyceride and LDL-C

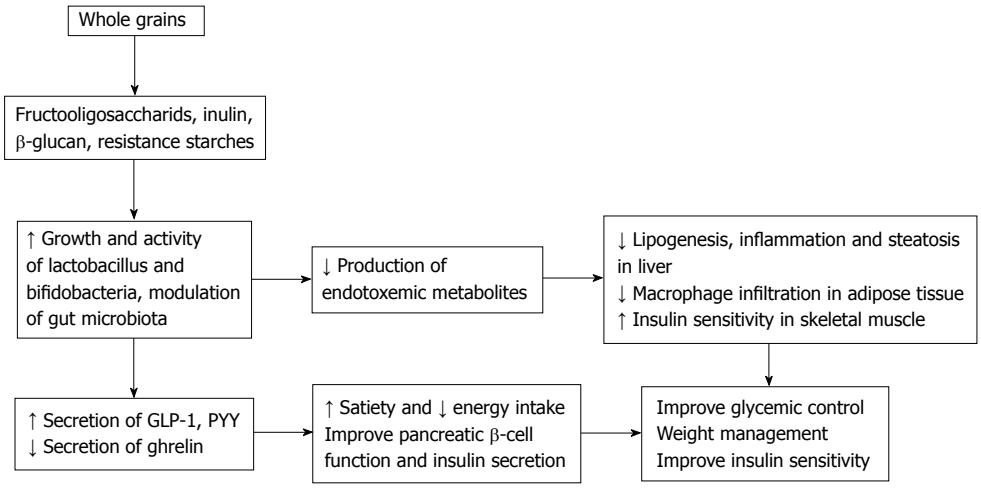

Figure 1 Role of prebiotic compounds of whole grains and cereal-based products in modulation of gut microbiota and con sequent metabolic effects could lead to better glycemic control.

levels, blood pressure and serum homocysteine levels, as well as vascular functions, and oxidative and inflammatory status[31].

Rye, a widely used grain especially in Northern and Eastern Europe, is considered a functional grain. The high fiber content of rye products decreases digestion and absorption of dietary carbohydrates, and increase metabolites derived from colonic fermentation of the soluble fiber of rye products, including propionic and butyric acids which effectively stimulate secretion of insulin from β-cells; studies have indicated that the bioactive compounds of rye (phenolic acids, tannins, benzoic acid, phenylalanine) derivates have a similar efficacy with antidiabetic drugs in insulin secretion[26,32]. In one study, the consumption of rye products in the breakfast meal increased colonic fermentation, decreased ghrelin levels and satiety rating in the late postprandial phase after breakfast as well as energy intake at a subsequent lunch meal, and improved acute glucose and insulin responses[32].

Oat meal products have also been investigated as healthy carbohydrate sources for diabetic patients; they are rich sources of soluble fiber especially β-glucan, antioxidants and bioactive compounds including carotenoids, phytic acid, phenolic acids (hydroxycinammic acids, caffeic acid, ferulic acid), flavonoids and phytosterols[33]. Studies show that consumption of oat products improves glycemic, insulinemic, and lipidemic responses in diabetic patients, and act as active ingredient reducing postprandial glycemia[34,35]. In diabetic animal models, oat products attenuated hyperglycemia-induced retinal oxidative stress, increased glycogen content of liver, decreased plasma free fatty acids and succinate dehydrogenase activity and inhibited pancreatic β-cell apoptosis as well[36].

The beneficial effects of barley and its by products for diabetic patients are mainly attributed to its high content of β-glucan; Administration of barley β-glucan extract in pre-diabetic subjects improved glucose tolerance and insulin resistance index[27]. In addition, barley may use as base of a meal; the use of barley combined with refined grains such as white rice maybe a practical way to attenuate their undesirable effects on glycemic control; in a randomized crossover study, combination of cooked barley with white rice dose-dependently reduced the area under the curves of plasma glucose and insulin concentrations, suppressed postprandial decrease of plasma desacyl ghrelin levels and consequently increased satiety[37]. The hypolipidemic properties, antioxidant and anti inflammatory activities of barley products have also been investigated[38,39]. In animal diabetic models, barley improved some features of fatty liver, decreased lipid content of the liver, increased fatty acid oxidation and adiponectin levels[40].

Several positive effects of whole wheat and its byproducts on carbohydrate and insulin metabolism have also been reported; wheat bran and whole wheat products are rich sources of dietary fiber, magnesium (main cofactor of enzymes involved in glucose metabolism and insulin secretion), potassium, phenolic acids, α-tocopherols, carotenoids and antioxidants[41]. It is believed that the majority of beneficial effects of whole wheat grain are related to bran and germ fractions; wheat bran is a main source of fiber, lignans, phenolic acid and alkylresorcinol, and beyond the health promotion of gastrointestinal tract and weight management, could improve postprandial glycemic response, glycosylated hemoglobin, lipid disorders and other cardiovascular risk factors in diabetic patients[42]. Studies showed that alkylresorcinol of wheat bran inhibited platelet activity and aggregation, decreased triglyceride de novo synthesis, and decreased cardiovascular disease risk factors[43]. Wheat germ is rich in non-digestible oligosaccharides, phytosterols, benzoquinone and flavonoids that play a potent role in induction of antioxidant and anti-inflammatory properties and modulation of immunity responses[44]. Avemar, fermented wheat germ extract, had interesting properties in the treatment of cardiovascular disease, and improved metabolic abnormalities including hyperglycemia, lipid peroxidation and abdominal fat gain[45].

Brown rice and its byproducts is another grain investigated as a functional food. Compared to white rice, brown rice has lower glycemic load and glycemic index, and higher content of fiber, vitamins and minerals, phytic acids, polyphenols, tocopherols, tocotrienols, and other bioactive compounds[46]; consumption of brown rice has benefits on glycemic control, dyslipidemia, endothelial function, abdominal obesity and liver functions in type 2 diabetic patients[47]. Studies show that γ-orizanol found in brown rice modulates high-fat diet induces oxidative stress, improves β-cell function, enhances glucose-stimulated insulin secretion and prevents the development of type 2 diabetes[48]. Germinated and pre-germinated brown rice, as more interesting functional foods, have unique components including γ-amino butyric acid, and bioactive acylated steryl glucosides with potent anti-diabetic properties; these bioactive components attenuate oxidative-induced peripheral nervous system, prevent diabetic neuropathy, inhibit oxidative-induced pancreatic β-cell apoptosis and enhance insulin secretion[49-51]. Bran rice, a byproduct of brown rice, contains within 31% fiber (mainly insoluble fiber), β-glucan, pectin, tocopherols, orizanol, ferulic acid, lutein, xanthine, vitamin K, thiamin, niacin, pantothenic acid, α-lipoic acid, coenzyme Q_{10} and other nutraceuticals; administration of bran rice in diabetic patients reduced glycosylated hemoglobin, LDL-C and total cholesterol as well as increased HDL-C[52].

In conclusion, replacement of whole grain and cereal-based products with refined grains in diet planning may be an effective and practical strategy for MNT in type 2 diabetic patients; this approach beyond the improvement of glycemic control, leads to more benefits for management of other aspects of diabetes, attenuation of diabetes-induced metabolic disorders, and prevents long-term complications especially atherosclerosis and cardiovascular disease.

PHYTOCHEMICAL-RICH FRUITS AND VEGETABLES

Fruits and vegetables are rich sources of dietary fiber (soluble and insoluble fiber), vitamins, and various phytochemicals and play a vital role in health promotion and prevention of chronic disease[53]. Dietary modification based on fruits and vegetables certainly is a definitely important strategy for management of type 2 diabetes and prevention of its complications; several studies indicate that regular consumption of various fruits and vegetables in diabetic patients can lead to an improved glycemic control, reduced HbA1c and triglyceride levels, enhanced antioxidant defense system, attenuated oxidative stress and inflammatory markers, decreased risk of diabetic retinopathy, and a lower burden of carotid atherosclerosis[54-57]. Since various fruits and vegetables provide many different micronutrients and bioactive compounds, consumption of varied fruits and vegetables is mainly recommended; it should be noted that the color of fruits and vegetables reflects predominant pigmented phytochemicals, and considering the colors in selection of these food groups provide a wide range of nutraceuticals. In Table 1, some phytochemical-rich fruits and vegetables, their bioactive compounds and favorable effects on diabetic related conditions are reviewed. Studies showed that tomato and its by products, as main sources of lycopene, β-carotene, flavonoids and other bioactive components, could attenuate blood pressure and dyslipidemia, decrease cardiovascular risk factors and enhance antioxidant defense system; other sources of lycopene and carotenoids such as grapefruit and watermelon have also beneficial properties to regulate lipid and lipoprotein metabolism, blood pressure and vascular function. Anthocyanins-rich fruits including red apple, berries family, grapes, cherries, red cabbage, and pomegranate have mainly hypoglycemic effects (↓ digestion and absorption of dietary carbohydrates, ↓ postprandial glycemic response and ↓ glycosylated hemoglobin) as well as protective properties against oxidative damages (Table 1).

LEGUMES

Legumes (peas, beans, lentils, peanuts) are valuable sources of dietary protein, non-digestible carbohydrates including dietary fiber, resistance starches, oligosaccharides, and bioactive compounds such as functional fatty acids (linoleic acid, α-linolenic acid), isoflavones (daidzein, genistein, glycitein), phenolic acids, saponins, and phytic acid; some polyphenols including pelargonidin, cyanidin, delphinidin, and malvidin are also found in legumes[134,135]. Legumes are considered a component of a healthy diet and there is much evidence showing that regular consumption of legumes has protective effects against obesity, type 2 diabetes, and cardiovascular disease[136]. Legumes may be considered as an important component of a functional-foods based diet for management of type 2 diabetes. α-amylase inhibitory peptides are one of the bioactive compounds in legumes and beans that reduce digestion and absorption of dietary carbohydrates, and modulate postprandial glycemic response; other bioactive peptides of grain legumes including the 7S globulin α chain and conglutin γ have unique properties to regulate lipid metabolism and normalize lipid and lipoprotein levels[137]. Low glycemic index, high fiber and phytochemical content of legumes have made them functional food for diabetic patients.

Lentils (Lens culinaris), the most consumed legume grains, are rich sources of dietary fiber, slowly digestible starch and resistant starch, tannins, β-glucan, functional antioxidant ingredients, a wide range of phenolic acids including gallic acid, proanthcyanidins, prodelphinidin, procyanidins, catechins, epicatechin, kampferol, quercetin, cinapic acid and apigenin[138]. Studies show that bioactive proteins of lentil reduce plasma levels of LDL-C, triglyceride content of the liver, and adipose tissue lipoprotein lipase activity; moreover, polyphenols of lentil could prevent angiotensin Ⅱ-induced hypertension, and pathological changes including vascular remodeling and

Table 1 Bioactive compounds and functional properties of some of favorable fruits and vegetables

Ref.	Possible functional properties in diabetes	Main bioactive components and phytochemicals	Fruits and vegetables
[58-62]	↓ Systolic and diastolic blood pressure ↑ apolipoprotein a1 and HDL-C ↓ LDL oxidation, improve diabetes-induced lipid disorders ↓ cardiovascular risk factors ↓ aldose reductase activity and cataract ↑ antioxidative enzymes activity	Lycopene, β-carotene, flavonoids, anthocyanins, phytoan, phyto flava, quercetin, kampferol	Tomato and its by products
[63-65]	↓ Triglyceride levels, enhance endogenous antioxidant defense system, regulation of appetite	Lycopene, pectin, naringin, hesperidin	Grapefruit
[66-69]	↑ Nitric oxide biosynthesis, improve endothelial function ↓ blood pressure ↑ plasma arginine levels and consequently ↓ insulin resistance and adipocyte size	Lycopene, carotenoids, cytrolin	Watermelon
[70-73]	↓ Absorption of dietary carbohydrate ↓ postprandial glycemia, improve pancreatic β-cell function ↓ free radical generation ↓ lipid peroxidation ↑ plasma total antioxidant capacity, prevent vascular damage, improve dyslipidemia	Soluble fiber, quercetin, catechins, epicatechin, *P*-cumaric acid, chlorogenic acid, gallic acid, phlordizin, procyanidins	Red apple, apple peel, apple and its by products
[74-81]	Glycemic control, inhibit α-glucosidase and α-amylase activity ↓ digestion and absorption of dietary carbohydrates ↓ insulin resistance, improve dyslipidemia ↓ postprandial oxidative stress ↓ lipid peroxidation ↑ plasma total antioxidant capacity ↓ systolic blood pressure ↑ antioxidative enzymes activity ↑ adipocytes lipolysis ↓ inflammatory processes, modulation of peroxisome proliferator-activated receptors	Anthocyanins, tannins, ellagitanins, α-carotene, β-carotene, lutein, delphinidins, pelargonidins, ciyanidins, catechins, hydroxy-cinnamic acid	Berries; cranberry, blackberry, black raspberry, blueberry, red raspberry, strawberries
[82-86]	Protective effects on vascular system ↓ platelet hyperactivity and aggregation ↓ cardiovascular diseases ↓ oxidative damage ↓ rennin-angiotensin activity ↑ production of nitric oxide ↓ blood pressure ↑ bone-marrow-derived endothelial progenitor cells	Anthocyanins, resveratrol	Grapes, grape by products
[87-91]	↓ Hyperglycemia ↓ HbA1c, improve lipid disorders, anti-inflammatory properties (inhibit cyclooxygenase) ↓ abdominal fat ↓ microalbuminuria, improve metabolic syndrome and fatty liver features ↓ oxidative stress ↓ production of cytokines, induction of PPARγ ↓ diabetic neuropathy	Anthocyanins, quercetin, hydroxy-cinnamic acid, carotenoids, melatonin, phenolic acids, gallic acid, lutein, xanthine, β-carotene	Cherries
[92-95]	↓ Hyperglycemia, attenuate hyperglycemia-induced metabolic disorders ↓ lipid peroxidation, induction of gluthathione reductase, glutathione peroxidase, superoxide dismutase, delay progression of nephropathy ↓ inflammatory processes, improve dyslipidemia	Isothiocyanates, anthocyanins (red cabbage), carotenoids, lutein, β-carotene	Cabbage, Cauliflower
[96-100]	↓ Hyperglycemia ↑ endothelial nitric oxide synthase activity, inhibit angiotensin converting enzyme ↓ blood pressure, improve vascular function ↓ cholesterol and atherogenic lipids ↓ lipid peroxidation ↓ progression of atherosclerosis ↑ plasma total antioxidant capacity, modulate activation of PPARγ and nuclear factor κB ↑ activity of paraxonase 1 and HDL-C levels ↓ serum resistin levels and ameliorate obesity-induced insulin resistance	Anthocyanins, tannins, catechins, gallocatechins, punicalagin acid, ellagic acid, gallic acid, oleanolic acid, ursolic acid, uallic acid	Pomegranate and its by products, pomegranate peel and seeds

[101-105]	↓ Hyperglycemia, induce insulin secretion from β-cell ↓ blood pressure, inhibit enzyme involved in cholesterol biosynthesis, improve dyslipidemia, prevent atherosclerosis ↓ lipid peroxidation ↓ platelet hyperactivity and aggregation, regulate glycolysis, gluconeogenesis and carbohydrate metabolism pathways ↑ insulin sensitivity	Allyl sulfors, flavonoids, quercetin, dihydroflavonols, anthocyanins (red onion)	Garlic, onions
[106-111]	↓ Endothelial macrophage activation ↓ hyperactivity and aggregation of platelet, improve vascular function ↓ oxidative stress and inhibit stress-sensitive signaling pathways ↓ digestion of dietary lipids, improve dyslipidemia ↓ pro-inflammatory cytokines ↓ lipid peroxidation	Lutein, xanthine, α-cryptoxanthin, β-cryptoxanthin, naringenin, hesperidin, β-carotene, phytosterols	Citrus fruits
[112-113]	↓ Free radical generation and lipid peroxidation, binding to bile acids ↑ cholesterol excretion, improve lipid profile ↑ plasma total antioxidant capacity	Lutein, betaine, violaxanthine, opioid peptides (rubisculins), *P*-cumaric acid, ferulic acid	Spinach
[114-115]	Improve glycemic and insulinemic response ↓ systemic inflammation ↓ cardiovascular disease risk factors	Carotenoids, pectin, oleic and linolenic acids	Pumpkin
[116]	Improve hyperglycemia and dyslipidemia ↑ adiponectin, antioxidant and anti-inflammatory effect	Fiber, polyphenols, chlorogenic acid, flavonoids, anthocyanins	Plums
[117-119]	Improve dyslipidemia, anti-inflammatory properties ↓ lipid peroxidation ↑ plasma total antioxidant capacity	Soluble fiber (pectin), α-carotene, β-carotene lutein, phenolic acids, stilbenes	Carrots
[120-122]	Inhibit α-amylase ↓ postprandial glycemia ↑ glycogen synthesis, improve dyslipidemia ↓ lipid peroxidation, protective effect against diabetic nephropathy	Carotenoids, quercetin, kampferol, gallic acid, caffeic acid, catechins, tannins, mangiferin	Mango
[123-127]	Regulate carbohydrate metabolism (↑ glucokinase and glucose-6-phosphate dehydrogenase activity ↓ glucose-6-phosphatase activity) ↓ lipid peroxidation ↓ protein carbonylation ↑ antioxidant enzyme activity, improve metabolic syndrome features ↑ insulin sensitivity ↓carbohydrate absorption ↓ plasma free fatty acid	Anthocyanins, alkaloid compounds (berberine, oxycontin)	Barberry
[128-131]	Protective effects against diabetic neuropathy ↓ lipid peroxidation, induce antioxidant enzymes, protect liver and kidney against oxidative damage	Dietary fiber, polyphenols, acid cinnamic, melatonin	Date fruit
[132-133]	Improve lipid and lipoprotein metabolism ↑ insulin sensitivity ↓ blood pressure	Dietary fiber, pectin, flavonoids, gallic acid, chlorogenic acid, catechins, anthocyanins	Figs

PPARγ: Peroxisome proliferator-activated receptor γ.

vascular fibrosis[139,140].

Beans are also other important legume grains in the human diet with high content of fiber, phytate, ω_3 fatty acids, antioxidants, phenolic compounds. The hypoglycemic effect of beans (*via* inhibition of α-amylase and β-glucosidase activity) has been reported as being similar to those of anti-diabetic drugs[141-143]. Including beans (pinto, dark red kidney, black beans) in diet planning for type 2 diabetic patients effectively helps weight management, attenuates postprandial glycemic response, and improves dyslipidemia[144-146].

Soybean, a rich source of unique phytoesterogens (genistein, daidzein, glycitein), is another important functional food which has been considered in diabetes; the isoflavones and bioactive peptides of soybean have favorable effects on glycemic control and insulin sensitivity, dyslipidemia, and kidney function[147-149]. It seems that the anti-diabetic effects of soybean mainly occur through interaction with estrogen receptors (ERs); studies show that soy isoflavones selectively bind to both α and β estrogen receptors; ERα is considered as key modulator of glucose and lipid metabolism, and regulate insulin biosynthesis and secretion as well as pancreatic β-cell survival[150]. Soy protein could induce insulin sensitivity and improve lipid homeostasis *via* activation of peroxisome proliferator-activated receptor and liver X receptors, and inhibition of the sterol regulatory element binding protein-1c[151]. Regular consumption of soy products could help diabetic patients in the management of dyslipidemia[152]. Soy protein and isoflavones decrease

production of atherogenic apolipoproteins such as apo B, increase biosynthesis of HDL-C, induce LDL-C receptors, increase biosynthesis and excretion of bile acids, decrease gastrointestinal absorption of steroids, induce favorable changes in hormonal status, including the insulin to glucagon ratio, and thyroid hormones which lead to improvement of dyslipidemia[153,154]. Recently two bioactive peptides, identified in glycinin (a main soy protein), have unique hypolipidemic properties. These peptides inhibit 3-hydroxy-3methyl glutaryl CoA reductase, key enzyme involved in cholesterol biosynthesis. β-conglycinin, another main soy bioactive protein with anti-atherogenic properties *via* regulation of lipogenesis, decease liver lipogenic enzyme activity, inhibits fatty acid biosynthesis in liver, and facilitates fatty acid β-oxidation; other biological activities of soy peptides include antioxidant, anti-inflammatory, and hypotensive effect[155].

Another feature of soybean and soy products as well as other legumes which may highlight them as main part of a functional foods-based diet, is their established effectiveness in weigh management; since the overweight and obesity are the common problems in diabetic patients and main contributors in development of insulin resistance, benefit from anti-obesity properties of legumes is considered another key approach in these patients. Thermogenic effects, induction of satiety through some important appetite regulatory gut peptides, mediation in gene expression and secretion of key adipocytokines such as leptin and adiponectin, as well as inhibitory effects on proliferation and differentiation of adipocytes are some of the mechanisms that could explain the role of legumes on weight management[140,156-159]. In conclusion, considering the potential benefits of legumes and its by products, regular consumption of these functional foods may be an effective strategy for management of various aspects of type 2 diabetes.

NUTS

Based on current evidence, nuts may play a protective effect against cardiovascular disease risk factors. Almonds, pistachios, walnuts and hazelnuts are commonly used nuts; these functional foods are considered as rich sources of high-biological value proteins, bioactive peptides, functional fatty acids (mono and poly unsaturated fatty acids), fiber, phytosterols, polyphenols, tocopherols and other antioxidant vitamins; the antioxidative effect of nuts mainly is related to a high content of α and γ tocopherol, phenolic acids, melatonin, oleic acid and selenium, while the anti-inflammatory effect is related to ellagic acid, α-linolenic acid and magnesium[160,161].

Most current evidence reveals that consumption of nuts in type 2 diabetic patients other than improving the overall diet quality also has beneficial effects on postprandial glycemic response following high-carbohydrate meals, attenuates postprandial oxidative stress and inflammatory processes, normalizes lipid and lipoprotein levels and decreases lipid atherogenicity, and improves insulin resistance[162,163]. Moreover, habitual intake of nuts could help to effectively manage weight especially in diabetic patients; the anti-obesity effects of nuts investigated in some studies may be attributed to thermogenic effects, induction of satiety, decreased dietary fat absorption, and increased fat excretion; bioactive components of nuts also modulate regulatory appetite neurotransmitters and adipose tissue metabolism, as well as decrease proliferation and differentiation of adipocytes, inhibit lipogenesis and induce fatty acid β-oxidation[164,165]. Studies show that consumption of nuts effectively decreases serum levels of high-sensitivity C-reactive protein; a well measure of systemic low-grade inflammation, interleukin 6 (a potent pro-inflammatory cytokine) and fibrinogen while increase plasma concentration of adiponectin, a potent anti-inflammatory cytokine released from adipose tissue; dietary patterns, high in nuts, were also related to lower levels of soluble inflammatory and cardiovascular risk markers including intercellular adhesion molecule 1 and vascular cell adhesion molecule 1[166,167]. Another beneficial effect of nuts which is important especially in diabetic patients is favorably influence on endothelial function; high content of L-arginine, a main precursor of nitric oxide, as well as antioxidants and polyphenols could contribute to this effect[161].

In conclusion, it seems that a diet enriched with nuts may be an effective strategy to improve glycemic control and prevent cardiovascular disease in type 2 diabetic patients.

OTHER BENEFICIAL FUNCTIONAL FOODS AND BIOACTIVE COMPONENTS FOR DIABETIC PATIENTS

Although there are a large number of natural foods, nutraceuticals or bioactive components that could be considered as functional ingredients and have beneficial effects for diabetes management, addressing all these issues is beyond the scope of this article. Table 2 shows some of these potential functional foods including dairy products and probiotics, fish meat, green tea, spices are presented.

CONCLUSION

Type 2 diabetes is a complicated metabolic disorder with both short- and long-term undesirable complications as well as various pathogenic conditions including dyslipidemia, vascular dysfunction, oxidative stress, sub-clinical inflammation, and altered signaling pathways. Ineffectiveness of the current medical treatments in management of long-term diabetes complications confirms that other complementary approaches are required; the use of functional foods and bioactive compounds is one of these new approaches. Functional foods and their bioactive compounds could attenuate carbohydrate metabolism and hyperglycemia, improve pancreatic β-cell function and insulin secretion as well as insulin resistance, regulate lipid and lipoprotein metabolism and adipose tissue metabolism, modulate oxidative/antioxidative balance and

Table 2 Bioactive compounds and functional properties of some of favorable functional foods

Ref.	Possible functional properties in diabetes	Main bioactive components and nutraceuticals	Functional foods
[168-179]	Improve the features of metabolic syndrome, modulate gut microbiota, regulate satiety and food intake ↑ adiponectin, modulate adipocytokines, induce thermogenesis, lipolysis and β-oxidation ↑ dietary fat excretion ↓ adiposity and body weight ↓ oxidative stress and inflammatory markers, hypo-lipidemic and anti-thrombotic effects ↑ insulin sensitivity, modulate immune responses in diabetic patients ↑ total antioxidant capacity ↓ lipid peroxidation ↓ HbA1c	Calcium, vitamin B, bioactive proteins such as casein and whey, immunoglobulines, bioactive peptides (α- and β-lactorphines, lactoferrin, lactoferricin, α-lactalbumin, β-lactoglobulin, growth factors), conjugated linoleic acids, lactic acid bacteria and bifidobacteria	Dairy products and probiotics
[180-185]	Improve hypertriglyceridemia and hypertension ↓ cardiovascular disease ↓ insulin resistance and inflammation, improve glycemic management ↓ proteinuria ↓ oxidative stress, inhibit lipogenesis and induce lipolysis, induce PPARα and PPARβ ↓ adiposity and weight management ↑ thermogenesis and energy expenditure, inhibit angiotensin converting enzyme and modulate blood pressure	Bioactive peptides, antioxidant compounds, ω_3 fatty acids (docosahexaenoic acid, eicosapentaenoic acid), selenium, taurine	Fish and seafood
[186-189]	Regulate cholesterol metabolism ↓ LDL oxidation, protect vascular endothelium against atherogenesis, inhibit platelet aggregation ↓ atherosclerosis development ↓ pro-inflammatory cytokines, activate PPARγ, improve sub-clinical inflammation	Oleic acid, ω_3 fatty acids, Flavonoids, cinnamic acid, benzoic acid, lignans, cumaric acid, ferulic acid, tocopherols, carotenoids, oleuropein, oleocanthal	Olive oil
[190-193]	Promote endogenous antioxidant defense system, induce superoxide dismutase and catalase ↓ lipid peroxidation, improve glycemic control ↑ insulin sensitivity ↓ gluconeogenesis ↑ glycogen content ↓ glycation of collagen and fibrosis, protect cardiac muscle, regulate lipid metabolism as well as adipose tissue metabolism, inhibit lipogenic enzymes ↓ satiety ↑ thermogenesis ↓ proliferation and differentiation of adipocytes ↓ pro-inflammatory cytokines ↓ monocyte chemotactic protein-1	Polyphenols, phenolic acids, catechins, epigallocatechin-3-gallat, chlorophyll, carotenoids, pectin, plant sterols	Green tea
[194-196]	↑ Iinsulin sensitivity, improve peripheral uptake of glucose, increase glycolysis and gluconeogenesis, hypoglycemic and hypolipidemic effects, antioxidant and anti-inflammatory properties	Cinnamaldehyde, cinnamic acid, coumarin, catechins, epicatechin, procyanidins B-2	Cinnamon
[197-199]	Inhibit enzymes involved in inflammation including cyclooxygenase-2, lipoxygenase, and nuclear factor κB, inhibit α-glucosidase and α-amylase activity ↓ postprandial glycemic response ↓ proteinuria, activate PPARγ and regulate carbohydrate and lipid metabolism, prevent diabetic cataract	Curcuminoids, stigmasterol, β-sitosterol, 2-hydroxy methyl anthraquinone, bioactive peptide turmerin	Turmeric
[200-203]	Attenuate oxidative stress, protective effects against oxidative damage ↓ serum creatinine and urea, improve dyslipidemia ↓ atherogenic lipoprotein levels ↓ lipid peroxidation in renal tissue, inhibit α-glucosidase activity ↓ carbohydrate digestion and absorption, protect liver against diabetes-induced oxidative damage	Tannins, flavonoids, anthocyanins, phenolic acid, gallic acid	Sumac

PPAR: Peroxisome proliferator-activated receptor.

inflammatory processes, improve weight management and prevent micro and macro vascular complications.

Considering the beneficial properties of functional foods, it seems that diet planning based on these healthy foods may be considered an effective strategy for management of various aspects of diabetes and promotion of health in diabetic patients.

ACKNOWLEDGMENTS

The authors express to acknowledge the assistance given by Ms Niloofar Shiva for critical editing of English gram-

mar and syntax. None of the authors had any personal or financial conflicts of interest.

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P- Reviewers: Hiroyuki T, Karoly R, Nahid P, Semir O
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
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ISBN 978-0-9861420-0-0

Social determinants of type 2 diabetes and health in the United States

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Received: December 18, 2013 Revised: April 19, 2014
Accepted: May 13, 2014
Published online: June 15, 2014

Abstract

Diabetes is the sixth leading cause of death in the United States. To date, most research and resulting clinical strategies have focused on the individual with short-term health improvements that have not been maintained over time. Researchers more recently have recognized the need to consider the social determinants of diabetes and health along with individual factors. The purpose of this literature review is to examine current understanding of the social determinants affecting diabetes and health. A search of medical and nursing literature was conducted using PubMed, PsychInfo, CINAHL and MEDLINE databases, selecting articles published between 2000 and 2013. Search terms included: type 2 diabetes, social determinants, and health determinants. Inclusion criteria were: English language, human studies, social determinants of diabetes and health, and research in the United States. Additional search methods included reference chaining of the literature. Twenty research articles met the inclusion criteria for the review and analysis and included quantitative and qualitative methods. All studies selected for this review were descriptive in nature (n = 20). Fifteen studies were quantitative studies and five were qualitative studies. No intervention studies met inclusion criteria. Each study is summarized and critiqued. Study findings indicate that external or upstream factors consistently affect individuals diagnosed with diabetes, influencing self-management. Significant methodological limitations result directly from small sample sizes, convenience or nonprobability sampling, and low statistical power.

Key words: Type 2 diabetes; Social determinants; Health determinants; Research; United States

Core tip: Social determinants of health and diabetes need to be considered when focusing on improving diabetes outcomes. Future research studies should focus on testing health outcomes of people with diabetes within the social determinants of health framework. Such research is particularly significant due to high rates of diabetes and subsequent disease sequelae.

Original sources: Clark ML, Utz SW. Social determinants of type 2 diabetes and health in the United States. *World J Diabetes* 2014; 5(3): 296-304 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/296.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.296

INTRODUCTION

Diabetes Mellitus affects approximately 25.6 million individuals or 11.3% of those over age 20. It is the sixth leading cause of death in the United States[1]. Diabetes places the individual at risk for serious long term complications including blindness, cardiovascular disease, end stage renal disease, hypertension, stroke, neuropathy, lower limb amputations, and premature death[1]. Estimated annual healthcare cost in 2012 for diabetes and its resulting complications was $245 billion[2]. Given the considerable differences internationally in methods of allocating health care resources, systems of funding and/or paying for care, and cultural attitudes to health and health care,

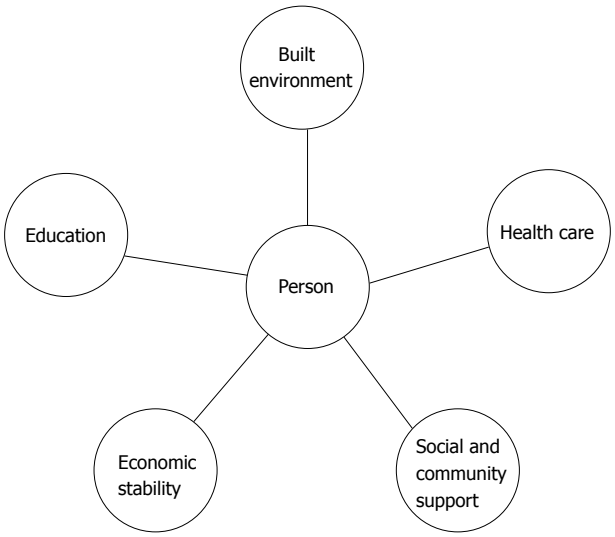

Figure 1 Social determinants influencing the individual's self-management of type 2 diabetes.

the purpose of this review of the literature is to examine current understanding of the social determinants affecting diabetes and health in the United States, and to make recommendations for future research.

Historically, research and resulting clinical approaches focusing on the individual have led to improvement in self-management outcomes and reduction of cardiovascular risk factors; however, these short-term improvements have not been maintained over time. Researchers more recently have recognized the need to consider factors external to the individual, namely the social determinants of diabetes and health in order to achieve the goal of sustainable improvement in health outcomes[3,4]. For example, the United States government document Healthy People 2020 emphasizes the social and environmental factors that affect the individual and his/her health. A Healthy People 2020 goal for the diabetes health indicator is to "reduce the disease and economic burden of diabetes mellitus, and improve the quality of life for all persons who have, or at risk for diabetes"[5].

Social determinants of health are social-ecological factors affecting health[6]. The person, his/her social network, and cultural and environmental conditions form the overall framework. Constructs include external/environmental socio-ecological influences on the individual (Figure 1); for example, culture, environment, education, working conditions, access to medical care, and community infrastructure[5]. Therefore, external or upstream determinants such as social support and elements of the community affect the health of the individual. Specific socio-ecological factors identified from this literature review are examined below.

Built environment/community infrastructure

Components of the physical environment include factors such as transportation, neighborhood safety, and healthy food. When barriers to these factors are present to individuals with diabetes, inadequate access to resources among such disadvantaged populations means fewer resources are available to overcome barriers, thus effects are magnified[7-9]. For example, limited transportation in rural areas may require travel outside the local community to gain access to healthcare providers or access to healthy foods[6]. Urban residents may face transportation barriers such as lack of sidewalks[9], discouraging individuals from walking as a form of physical activity. Lack of public transportation in rural or urban areas can hinder travel for access to healthcare. Lack of neighborhood safety contributes to health disparities. An example of compounding factors is as follows: urban centers may have high crime rates with consequently fewer businesses and employment; reduced access to services including food and medical care; and diminished opportunity for outdoor activity including exercise[10]. Research has shown a relationship between improved health outcomes and access to healthy foods[11,12]. Emerging research in the area of nutraceuticals indicates that certain foods may provide health benefits to reduce disease process progression in diabetes and hyperlipidemia[12]. However, this relationship is a complicated one, as demonstrated by Jones-Smith *et al*[13] who found that, even with access to healthy food, socioeconomic status remains a strong predictor for obesity among African Americans diagnosed with diabetes.

Economic stability

Research has demonstrated a direct relationship between socio-economic status and health outcomes; however, other factors may explain a degree of variance in this relationship[14]. Zheng *et al*[14] found that education level, employment, and family income affect socioeconomic status and therefore health.

Education

Greater educational attainment has been linked with improved health outcomes[15] possibly because of a greater likelihood of socio-economic stability compared to those with lower levels of education. Other related factors may be the stability derived from marriage and/or a wider range of opportunities for better employment[15]. Moreover, research has shown that individuals with higher levels of education are more likely to participate in preventive healthcare including eating healthier (foods), being more physically active, and avoiding obesity[16].

Health care/access to medical care

Individuals may be subject to disparity in the availability of healthcare resources, including access to medical care, based on factors such as socioeconomic status, place of residence, race/ethnicity, and culture. Socioeconomic factors include educational level which in turn influences health insurance status[16]. Low income inner cities and remote rural regions often lack both primary and specialty healthcare providers, decreasing access to healthcare for inhabitants with chronic illnesses such as diabetes, hypertension, and cardiovascular disease. Absent or inadequate care may result in worsening or compounding of long-term effects of chronic diseases[17,18]. For example, recent

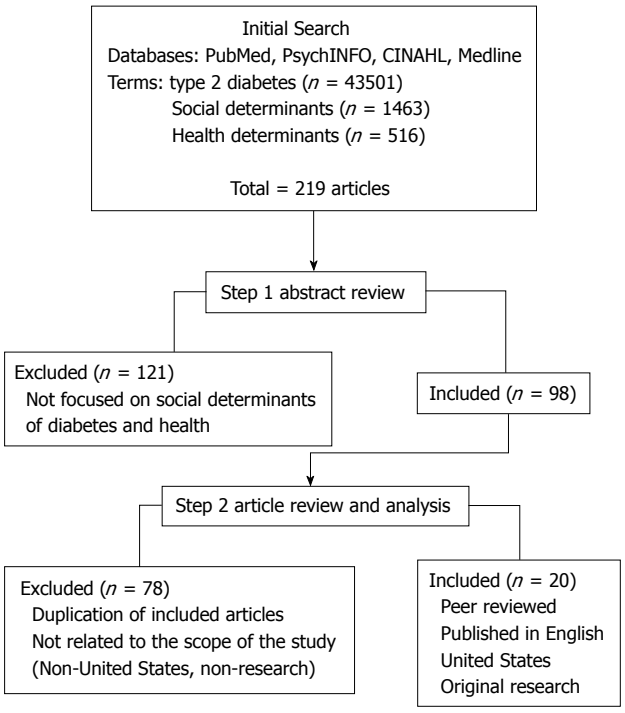

Figure 2 Manuscript selection for systematic review of the social determinants of diabetes and health.

research focusing on infants born preterm or with low birth weight demonstrates an increase in the development of insulin resistance and impaired glucose tolerance as adults[19,20]. Lower socioeconomic status may be associated with an individual's perception that lack of a collaborative patient-provider relationship is associated with improved diabetes outcomes[21].

Culture/social and community support

Social support includes individuals' "formal and informal relationships that give rise to a belief that one is cared for or supported emotionally in a defined situation such as working toward improving health outcomes"[22]. Degree of social support may vary between individuals and among ethnic groups; for example, research revealed that Hispanic individuals diagnosed with diabetes prefer group medical visits for self-management support whereas individuals from other ethnic groups have no preference[23]. Just as greater social support correlates with improved self-management outcomes, the perception of negative or low levels of social support has been shown to increase the risk of fewer self-management behaviors[24].

RESEARCH

A search of medical and nursing literature was conducted using PubMed, PsychInfo, CINAHL and MEDLINE databases. Additional search methods included reference chaining of the literature. Search terms included type 2 diabetes, social determinants and health determinants. Inclusion criteria were English language, human studies, social determinants of diabetes and health, and research in the United States. Exclusion criteria were type 1 diabetes, reviews, and studies not focusing primarily on social determinants of diabetes and health; for example, biomarkers. The initial search of the literature retrieved 59036 articles on type 2 diabetes; 12871 articles on social determinants; 14866 articles on health determinants. Sixty one duplicate articles, one book review brief, one editorial commentary, and two conference proceeding abstracts were also excluded (Figure 2). Twenty articles met criteria for the review (Table 1).

Twenty articles met the inclusion criteria for the review and analysis. All studies selected for this review were descriptive in nature (n = 20). Fifteen studies were quantitative studies and five were qualitative studies[25-29,32-44]. Although sample size ranged from 15 to 81917 participants, many samples were fewer than one hundred subjects. All studies focused on individuals diagnosed with diabetes. There were no interventional or randomized control trial studies. The majority were cross-sectional, collecting data only once. For quantitative studies, two were mixed methods, including a survey and interview; five were secondary data analysis, and eight were surveys. Qualitative studies used either focus groups or individual semi-structured interviews (n = 5). Fourteen studies focused on social determinants from the patient or client perspective; three studies focused both on staff/healthcare provider and patient/client, while three studies viewed social determinants of health from the perspective of the healthcare provider alone. All studies focused on one or more of the constructs of social determinants of health: built environment, economic stability, health care, or culture/social support.

Built environment/community infrastructure

Authors of four articles discussed the built environment and community infrastructure. Research studies used purposive sampling, limiting the generalizability of findings to other populations. Three studies focused on populations known to have a disproportionate burden of type 2 diabetes, including African Americans and Hispanic/Latino. The built environment was a stronger predictor of health outcomes than race. Three studies[25-27] reported on upstream social determinants and the influence on food environments for at risk immigrant Hispanic population. Findings included high rates of poverty with 60% of living below United States definition of poverty and 40% living at 170% below federal poverty level. Educational attainment was less than the United States average with 80% of individuals not entering college. Thirty-three percent had not completed elementary school. In comparison the national United States rate of high school completion is 89.9% in 2010[25]. One study focused on Asian Americans. No studies included American Indians or Pacific Islanders. Two studies were community-based, focusing on food environment and access to healthy food. Transportation was discussed in three articles as a barrier to access both healthcare and healthy food. Research participants reported lack of access to quality, quantity, and

Table 1 Summary of reviewed studies

Ref.	Purpose	Design/method	Sample	Findings	Strengths/limitations/implications
Chaufan *et al*[25] (2011)	To examine "upstream" social determinants of health in a Latino immigrant population focusing on T2DM risk and food environment	Mixed methods focus groups, Food store survey instrument California	Staff members (n = 6); clients (n = 15)	Poverty prevented Latino immigrants from access to adequate housing, high quality education, and culturally appropriate food choices	Community-based interviews elicited lived experience of Latino immigrant population Limitations: small sample size, nonrandomized, descriptive study Implications: Policy change to enable access to affordable culturally acceptable healthy foods
Carbone *et al*[26] (2007)	To describe factors influencing Latino (Puerto Rican) diabetes self-management	Qualitative-focus groups Health center in Massachusetts, United States	Healthcare providers (n = 15) Patients (n = 37)	Cultural influence of family through traditional gender roles; financial restraints Social network (family and friends) source of strength; spirituality and faith important; discrepancy between healthcare provider and patient focus in self-management	Community-based limitations: Small sample size, qualitative study, not generalizable to all Latino populations Implications: account for inclusion of family and social networks in diabetes self-management education, need for increased cultural awareness by Healthcare provider of influencing factors on patient self-management
Chaufan *et al*[27] (2011)	To gain understanding how food environments influence a low income immigrant population	Qualitative-focus groups	Staff (n = 6) Clients (n = 15)	Barriers to food access: transportation, language barriers, poverty, employment	Strengths: community based Limitations: small sample size, not generalizable Implications: Need for policy to increase food access for immigrant populations in lower socio-economic levels
Tjia *et al*[28] (2008)	To gain understanding of barriers to medication adherence among older adults with T2DM	Qualitative-semi structured interviews	Adults over age 65 (n = 22) Female n = 16	Older adults with T2DM had concerns about medication cost or medication burden but did not discuss with physician	Strengths: perspective of older adults Limitations: small sample size, not generalizable Implications: need for increased patient-physician communication; policy level for messages to encourage open patient-physician dialogue for medical treatment
Denham *et al*[29] (2010)	To explore perceived patient barriers to diabetes education among healthcare providers	Cross-sectional survey	Healthcare providers from three practice settings: federally qualified healthcare centers; health departments; clinics (n = 182)	Perceived barriers: transportation; fewer diabetes educators and physicians; lack of insurance; education materials not screened for literacy levels nor cultural appropriateness	Strengths: look at perceived barriers to diabetes education among healthcare providers Limitations: small sample size, multiple sites, sample bias Implications: policy need to increase diabetes education reimbursement to providers for all individuals nationally; need to screen materials for literacy levels
Heuer *et al*[30] (2006)	To describe Hispanic migrant farmworkers perceptions of diabetes	Qualitative phenomenological study	Migrant farmworkers (n = 12) Female n = 6	Cultural/folk beliefs that diabetes was caused by stress or emotions	Strengths: focused on hispanic explanatory model of diabetes Limitations: small sample size, not generalizable Implications: need to tailor diabetes education to address culture and health beliefs
Shigaki *et al*[31] (2010)	To examine how patients diagnosed with type 2 diabetes view role of nurses in disease management	Qualitative Semi-structured individual interviews	n = 13 Female n = 7 White n = 9 African American n = 3 Other n = 1	Nurse viewed as a positive partner in disease management, patients prefer team-based medical care, open communication between healthcare providers and patient	Strengths: Patient perspective on nurse as partner in healthcare Limitation: small sample size; sample bias Implications: Nurse partner may provide link between patient and improving health outcomes in individuals with multiple co-morbidities including diabetes
Fitzgerald *et al*[32] (2008)	To determine diabetes care perceptions in patient and provider	Quantitative cross sectional survey	Providers n = 71 Patients: n = 273 female 61% White 63% African American 33%	Patient and provider differences included patients with more positive attitudes family support, paying for diabetes care	Strengths: quantitative study using reliable and valid instrument Limitations: may not be representative for patients with no co-morbidities Implications: Importance of communication between patient and provider and not making assumptions about patients

Zulkowski *et al*[33] (2005)	To examine differences between patient self-report and provider documentation in rural health center	Quantitative cross sectional survey; medical record review	*n* = 149 Female *n* = 86	Statistical difference between patient and provider on medical management or diabetes knowledge; most patients had not been seen by diabetes educator or dietician	Strengths: good response rate (61%); limitations: race not documented Implications: Need for increased access to diabetes education in rural areas; need for open communication between patient and provider
Ford *et al*[34] (2002)	To determine relationship between socio-economic status and race on diabetes management	Quantitative-random sampling by socio-economic status and race	*n* = 50 African American *n* = 21 Caucasian *n* = 29 Female *n* = 22	Differences in perception of sense of loss by race but not socio-economic status	Strengths: Limitations: small sample size: not generalizable; underpowered (α = 0.34) Implications: Need for education to stress management to prevent disease related complications
Mani *et al*[35] (2011)	To determine the influence of social networks on diabetes self-management	Cross-sectional survey	*n* = 154 Female *n* = 88 White *n* = 78 African American *n* = 61	Diabetes concern increased when larger social network diagnosed with diabetes	Strengths: patient perspective of importance of social networks Limitations: not generalizable-sample from two inner city clinics, only English speaking, data skewed Implications: Important to consider the influence of social networks on diabetes self-management
Song *et al*[36] (2012)	To examine social networks among Korean Americans diagnosed with type 2 diabetes	Cross-sectional survey from a larger study	*n* = 83 Female *n* = 35 Married *n* = 73	Gender differences in source of support, men sought spouse, women had higher unmet needs for social support than men, self-efficacy negatively associated with unmet social support, education level strong predictor of self-care activities, unmet needs for social support negatively associated with diabetes self-care	Strengths: patient perception of social support Limitations: small homogenous sample; not generalizable Implications: need to recognize the role of gender in determining social support among Korean Americans
Kollannoor-Samuel *et al*[37] (2011)	To identify influence of social determinants of health on FPG and HbA1c among low income Latinos	Cross-sectional	*n* = 211 Female *n* = 155 Puerto Rican *n* = 171 Unemployed *n* = 178 Spanish speaking only *n* = 138	Lower socio-economic status had higher FPG and HbA1c levels; better long term glycemic control when insured; increased physical activity associated with lower FPG and HbA1c levels	Strengths: sample size; part of larger RCT Limitations: large number of female participants-not generalizable or transferable to other Latino populations; unknown length of time since diagnosis Implications: Importance of physical activity for improved glycemic control; need for health insurance, increase in education to improve glycemic outcomes over the long term
Chiu *et al*[38] (2011)	To determine how gender influences functional limitations with type 2 diabetes	Secondary data analysis	*n* = 1619 Female *n* = 861	Psychosocial factors mediator between biological and exercise factors	Strengths: consistent with previous research Limitations: participant self report; no causal pathway Implications: Need to incorporate gender and psychosocial factors in diabetes treatment plan
Iida *et al*[39] (2010)	Examine role of spousal support for individual diagnosed with type 2 diabetes	Mixed methods-longitudinal Computer diary survey; individual interviews	*n* = 129 married couples Caucasian 75%; African American 23.6%	Physical symptoms increased spousal support; women gave higher level of support when spouse worried about diabetes; less spousal support when negative emotional effect from previous day	Strengths: sample size Longitudinal study, diary Limitations: unable to determine causal effect Implications: Need to measure and include spousal support in diabetes self-management plan

Mayberry *et al*[40] (2005)	To assess determinants of glycemic monitoring by primary care provider among medicaid beneficiaries	Retrospective cohort study	*n* = 3321 African American (2025) 61%; White (1296) 39%; Female 74.6%; Urban 49.6%; rural 50.4%	African Americans diagnosed at younger age; frequency of physician visits and medication prescription strong predictor of meeting ADA recommendation guideline for HbA1c monitoring; low monitoring of HbA1c and FPG among medicaid beneficiaries with type 2 diabetes, no significance between Black and White Medicaid beneficiaries for diabetes monitoring	Strengths: provides insight into determinants of glucose monitoring in Medicaid beneficiaries Limitations: Secondary data analysis; inability to determine patient-physician relationship Implications: Need to establish ADA evidence guidance protocol in office practice settings
Ford *et al*[41] (2005)	To determine estimates of obesity and diabetes weight information from healthcare providers in 100 United States metropolitan service areas	Cross sectional survey: 2000 Behavioral Risk Factor Surveillance System	*n* =81917 individuals with BMI data Sample size varied due to missing data	Highest prevalence of obesity Appalachian region; OR of being diagnosed with diabetes 3.5 higher in Charleston WV than Santa Fe NM; weight loss or maintenance discussed with physician 11.7%-34.6%	Strength: study at community level Limitation: secondary data analysis, missing data; self report Implications: Policy level- need to examine determinants of obesity and diabetes at local metropolitan service areas. Healthcare providers need to include discussion of weight management for individuals diagnosed with obesity or diabetes
Adams *et al*[42] (2008)	To examine medication adherence as a factor of glycemic control based on race	Secondary data analysis newly diagnosed patients prescribed oral medications	*n* = 1806 Black *n* = 467 White *n* = 1339	Black patients higher A1c at diagnosis; insufficient evidence to determine medication adherence by race	Strengths: limitations: unable to determine causal effect; potential overestimation of medication adherence based on data; implications: Need at policy level to provide screening for earlier diagnosis of Black and female patients
Aikens *et al*[43] (2005)	To examine the relationship of patient-provider communication on diabetes self-management and outcomes	Cross-sectional Telephone survey	*n* = 736 White 51% Black 20% Hispanic 11.9% Female 31.6%	Primary care provider main manager of diabetes (70.9%) General patient-provider communication related to improved quality of life; diabetes specific patient-provider communication related to glycemic control	Strengths: sample across three health systems; limitations: cross-sectional study; self-report; implications: need for open patient-provider communication to enhance problem solving, need to tailor management plan to individual patient
Paris *et al*[44] (2001)	To determine if determinants of type 2 diabetes were present in personnel entering the military	Cross-sectional secondary data analysis	Diagnosed with diabetes *n* = 419 Not diagnosed with diabetes *n* = 627	Enlisted military rank as socioeconomic measure for diabetes diagnosis higher in lower rank, minorities higher level of diabetes; educational not significant variable	Strengths: adequate power Limitations: no causal effect; potential for misclassification of diabetes diagnosis in database; implications: need for focus on policy level for increased physical activity and body mass index monitoring

FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin A1c; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; RCT: Randomized control trial; ADA: American diabetes Association.

culturally-acceptable food choices[25-27]. Study limitations include small sample size and descriptive statistics.

Economic stability

Five articles focused on economic stability. Three studies were cross-sectional survey, one involved focus group interviews, and one was a secondary analysis. Sample size in these studies ranged from 50 to 419. Discussion focused on health insurance, financial barriers, poverty, and affordability of medication from the patient or client perspective. One study focused on indirect economic factors, such as military rank, as a predictor of diabetes diagnosis[44]. Two studies compared patient/client perspectives to healthcare provider perceptions of economic barriers to diabetes self-management[25,32]. Three of the five studies compared race as a factor in economic stability within Latino and African

American populations[26,34,37]. Findings included individual acknowledgement that economic distress in diabetes self-management was important however, factors were also identified as sources of additional strength for individuals diagnosed with diabetes. Sources of support included culture and/or social support[26,34]. The influences of economic factors by race/ethnicity on diabetes outcomes were non-conclusive. Therefore, economic stability may be a strong determinant of diabetes and health regardless of race/ethnicity. Studies focused on target populations, limiting to selected urban regions for study.

Health care/access to medical care

There were nine studies found in which researchers examined the role of health care and/or access to medical care within the social determinants of diabetes and health framework. One study compared patients' and healthcare providers' perspectives on diabetes management[32]. Another research report examined healthcare providers' perception of patient barriers to diabetes management[29]. The remaining seven articles focused on the patient's perceptions of healthcare related to diabetes management and barriers to care. Sample size for the patient-only studies ranged from 13 to 81917. Eight studies were cross-sectional descriptive in design, and one was a secondary data analysis from the Behavioral Risk Factor Surveillance System. Most researchers reported that patients viewed their health in a more positive light than did providers based on medical record reviews. The concerns of healthcare provider included the costs associated with diabetes management[29,32]. Patient-provider communication varied among patients. Three articles focused on positive health outcomes with open patient-provider communication[31,40,43]. One article described physicians as often initiating communication about medication adherence, whereas patients were hesitant to initiate communication with physicians relating to medication burden and costs[28]. This may, in part, explain perceived lack of patient medication adherence which increases the potential for poorer health outcomes. One qualitative study described patients' preference for diabetes care teams in which the team's link between patient and physician was a nurse[31]. Two studies demonstrated increased quality of life and better glycemic control with positive patient-provider communication[40,43]. However, when looking at diabetes prevention and knowledge, two studies reported the need for provision of diabetes education focusing on basic management and the need for discussion of weight management or weight loss for diabetes prevention[33,41].

Culture/social and community support

Seven articles met the inclusion criteria focusing on the constructs of culture and community support. Four of the seven researchers reported on cross-sectional surveys, one study involved focus groups in a community setting, one study used a phenomenological method of analysis, and one used mixed methods incorporating a computer diary and individual interviews. Two of the seven articles included healthcare provider perceptions. Of these two articles, one had a sample of both patient and healthcare provider. Sample size for the seven articles ranged from 12 to 273. Two articles focused on cultural determinants of diabetes and health in Latino/Hispanic populations. Cultural beliefs in Hispanic populations included the belief that diabetes was caused by increased stress[30]. The authors noted that the discovery of this belief provides an opportunity for healthcare providers or trusted community sources to provide education to increase diabetes knowledge. Three articles focused on the traditional roles of gender and culture, whereby married women provided increased support to their spouse when he voiced concerns about diabetes and health[26,36,39]. One article focusing on Korean Americans found that women had an increase in unmet needs when providing support for their spouses, which negatively affected their diabetes self-care[36]. Two articles discussed social support or social networks as positive influences for diabetes self-management and health[26,32]. However, one article described African American patients' concern about their diabetes management and health when multiple members of their social network were diagnosed with diabetes or experienced complications of diabetes[35]. One article discussed healthcare providers' perceived barriers in rural healthcare settings[29], pointing out an apparent lack of culturally appropriate educational materials within healthcare clinic settings.

CONCLUSION

This critique of the literature about social determinants of diabetes and health focused on research of United States populations published between 2000 and 2013. A total of 20 research studies met established criteria. All 20 studies identified for this review were descriptive. The majority of studies were published in journals with a focus on public health or nursing. Results of this review are useful for health professionals who develop programs and/or interventions for people diagnosed with diabetes because evidence indicates that social determinants affect patient adherence, effectiveness of treatments, and overall health outcomes.

Study findings indicate that external or upstream factors prominently affect individuals diagnosed with diabetes, in part by influencing self-management and in turn exerting lasting effects on long-term diabetes and health outcomes. The most significant methodological limitations of the studies examined result directly from small sample size, convenience or nonprobability sampling, and low statistical power. Methodological limitations of studies included in this review also include a lack of intervention studies. Future research needs to include community-based intervention studies focusing on the reduction of diabetes disparities and improvement of health outcomes within the social determinants of health framework. Such research is particularly needed given the high rates of diabetes and subsequent disease sequelae. Cultural tailoring

of diabetes prevention educational materials and cultural tailoring of education in group settings may afford the means to increase patients' knowledge of the disease for earlier diagnosis and earlier intervention to prevent diabetes complications. Encouragement of spousal support within the construct of acknowledging cultural norms may provide a means for improving diabetes outcomes and health. The influence of social determinants of health on diabetes outcomes needs to be tested in intervention studies to provide a foundation for effective interventions to impact the current epidemic of diabetes in the United States and around the globe. Prospective interventional studies evaluating the influence of social determinants will be key to lay a foundation for effective interventions and improvement of diabetes and health outcomes.

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P- Reviewers: Ciccone MM, Cui WP **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Novel and emerging diabetes mellitus drug therapies for the type 2 diabetes patient

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Received: December 9, 2013 Revised: January 24, 2014
Accepted: April 3, 2014
Published online: June 15, 2014

Abstract

Type 2 diabetes mellitus is a metabolic disorder of deranged fat, protein and carbohydrate metabolism resulting in hyperglycemia as a result of insulin resistance and inadequate insulin secretion. Although a wide variety of diabetes therapies is available, yet limited efficacy, adverse effects, cost, contraindications, renal dosage adjustments, inflexible dosing schedules and weight gain significantly limit their use. In addition, many patients in the United States fail to meet the therapeutic HbA1c goal of < 7% set by the American Diabetes Association. As such new and emerging diabetes therapies with different mechanisms of action hope to address some of these drawbacks to improve the patient with type 2 diabetes. This article reviews new and emerging classes, including the sodium-glucose cotransporter-2 inhibitors, 11β-Hydroxysteroid dehydrogenase type 1 inhibitors, glycogen phosphorylase inhibitors; protein tyrosine phosphatase 1B inhibitors, G Protein-Coupled receptor agonists and glucokinase activators. These emerging diabetes agents hold the promise of providing benefit of glucose lowering, weight reduction, low hypoglycemia risk, improve insulin sensitivity, pancreatic β cell preservation, and oral formulation availability. However, further studies are needed to evaluate their safety profile, cardiovascular effects, and efficacy durability in order to determine their role in type 2 diabetes management.

Key words: Type 2 diabetes mellitus; Sodium dependent glucose co-transporter 2 inhibitors; 11β-Hydroxysteroid dehydrogenase type 1 inhibitors; Glycogen phosphorylase inhibitors; Protein tyrosine phosphatase 1B inhibitors; G protein-coupled receptor agonists; Glucokinase activators

Core tip: Type 2 diabetes mellitus is a metabolic disorder of deranged fat, protein and carbohydrate metabolism resulting in hyperglycemia. Limited efficacy, adverse effects, cost, contraindications, renal dosage adjustments, inflexible dosing schedules and weight gain significantly limit the use of currently available anti-hyperglycemic agents. In the past, drug researchers targeted defects of pancreatic β-cell failure and insulin resistance, but more recent attention has shifted to other contributing factors. This article reviews new and emerging diabetes classes, including the sodium-glucose cotransporter-2 inhibitors, 11β-Hydroxysteroid dehydrogenase type 1 inhibitors, glycogen phosphorylase inhibitors, protein tyrosine phosphatase 1B inhibitors, G protein-coupled receptor agonists, and glucokinase activators.

Original sources: Rochester CD, Akiyode O. Novel and emerg-

ing diabetes mellitus drug therapies for the type 2 diabetes patient. *World J Diabetes* 2014; 5(3): 305-315 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/305.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.305

INTRODUCTION

Type 2 diabetes mellitus is a metabolic disorder of deranged fat, protein and carbohydrate metabolism resulting in hyperglycemia from insulin resistance and inadequate insulin secretion, which can cause complications of nephropathy, retinopathy, neuropathy, and cardiovascular disorders[1,2].

Diabetes mellitus is an epidemic in the United States and the world. According to the International Diabetes Federation's 2013 statistics, 382 million people worldwide have diabetes, which is estimated to increase to 592 million by 2035[3]. The Centers for Disease Control and Prevention estimates 79 million Americans have pre-diabetes and approximately 26 million have diabetes mellitus of which seven million of these are still undiagnosed[4].

Despite a wide variety of available food and drug association (FDA) approved oral and injectable diabetes therapies, limited efficacy, adverse effects, cost, contraindications, renal dosage adjustments, inflexible dosing schedules and weight gain significantly limit their use[5,6].

In addition, less than 50% of patients with type 2 diabetes in the United States achieve the HbA1c goal of < 7% set by the American Diabetes Association[7].

Currently available oral agent classes include sulfonylureas, meglitinides, biguanide, α-glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, dopamine agonist, bile acid sequestrant, thiazolidinediones and their combinations. Injectable agents include insulin, amylin analogue and incretin mimetics.

In the past, drug researchers and manufacturers targeted the primary pathophysiologic defects in type 2 diabetes of pancreatic β-cell failure and insulin resistance, but more recent attention has shifted to other contributing factors including increased glucose reabsorption by the kidneys, and the contributing effects to hyperglycemia by glucagon, glucocorticoid, glycogen, 11β-Hydroxysteroid dehydrogenase-2 and others. As such new and emerging diabetes therapies with new mechanisms of action hope to address these contributing pathophysiologic defects and offer new approaches in order for the patient to achieve therapeutic goals[1,6]. Table 1 lists the new and emerging drug therapy and approaches[8].

An ideal antihyperglycemic agent will be a safe, tolerable, efficacious, cost effective oral agent with a flexible dosage schedule providing clinically significant weight loss with cardiovascular and mortality benefits. This article reviews several new classes of antihyperglycemic agents, including the sodium-glucose cotransporter-2 inhibitors (which are furthest along in development); 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD-1) inhibitors, glycogen phosphorylase inhibitors, protein tyrosine phosphatase 1B inhibitors, G Protein-Coupled receptor agonists and glucokinase (GK) activators.

SODIUM DEPENDENT GLUCOSE CO-TRANSPORTER 2 INHIBITORS

Kidney and sodium dependent glucose co-transporter 2 transporters

Glucose homeostasis involves the liver, pancreas and the kidney[9]. Glucose transporter proteins (GLUT) and sodium-dependent glucose co-transporters (SGLT) are responsible for glucose transportation across the plasma membrane into cells[10].

Over the course of 24 h, the kidney filters 180 g of glucose while only 500 mg is excreted in the urine, and the rest is reabsorbed as it flows from the glomerulus to the proximal convoluted tubules then to the bloodstream[10]. GLUTs and SGLTs are involved in this glucose reabsorption and active transportation of glucose across cell membranes against concentration gradients[10,11].

SGLT-1 is responsible for 10% of glucose uptake and is expressed in the heart, skeletal muscle, gastrointestinal tract, liver, lung and the S3 segment of the proximal tubule of the kidney, while SGLT-2 is responsible for 90% of glucose uptake and is expressed in the S1 segment of the proximal tubule of the kidneys[11,12].

In addition to the reabsorption of approximately 99% of glucose, recent studies show the kidney takes up lactate, glutamine, glycerol, and alanine and converts them to glucose by the process of gluconephrogenesis, which can account for about 20% of all glucose released into the circulation and nearly 90% of the glucose released by the kidney[13].

The SGLT-2 inhibitors inhibit SGLT-2, which increases renal excretion of glucose thus reducing glucose in the plasma. Due to the minimal glucose uptake by SGLT-1 and the important roles of SGLT-2 in glucose reabsorption, several researchers and manufacturers have turned their attention to SGLT-2 inhibitors for treating hyperglycemia[14-16]. There are several SGLT-2 inhibitors in varying phases of studies including dapagliflozin, empagliflozin, ipragliflozin, ertugliflozin, luseogliflozin, tofogliflozin and LX4211[6,17].

The FDA approved canagliflozin (Invokana®) to treat type 2 diabetes based on the agreement that post marketing studies will be completed for evaluating cardiovascular outcomes, malignancies, severe pancreatitis, hypersensitivity and photosensitivity reactions; liver abnormalities, adverse events during pregnancy, bone safety, and two pediatric studies under the Pediatric Research Equity Act CR[18].

Dapagliflozin was approved in Europe, Australia, Brazil, Mexico and New Zealand as Forxiga®, but the FDA initially delayed its approval as there were concerns of increased breast and bladder cancer in patients taking the drug compared to placebo[19].

In January 2014, the FDA approved dapagliflozin as Farxiga® with six postmarketing studies including a

Table 1 Emerging classes of medications and approaches[8]

SGLT inhibitors
11β-HSD-1 inhibitors
GKA
AMPK agonists
SIRT activators
PTP-1B inhibitors
GCGR antagonists
GR antagonists
Novel insulin sensitizers
GPR119 agonists
Other drugs augmenting GLP-1 secretion: GPR40, G-protein coupled bile acid receptor (TGR5) agonists
Acyl-CoA: DGAT1 inhibitors
FGF-21-receptor agonists
Ranolazine
Other glucometabolic approaches
Other metabolic approaches
Anti-inflammatory approaches
Induction of immune tolerance
Pancreatic beta cell protection and regeneration
Pancreatic islet cell transplantation
Various antidiabetic approaches

SGLT: Sodium-dependent glucose co-transporter; 11beta-HSD-1: 11beta-hydroxysteroid dehydrogenase type 1; GKA: Glucokinase activators; AMPK: Adenosine monophosphate activated protein kinase; SIRT: Sirtuin; PTP-1B: Protein tyrosine phosphatase-1B; GCGR: Glucagon receptor; GR: Glucocorticoid receptor; GPR119: G-protein coupled receptor 119; GLP-1: Glucagon like peptide-1; Acyl-CoA: Acyl-coenzymeA; DGAT1: Diacylglycerol acyltransferase1; FGF-21: Fibroblast growth factor-21.

cardiovascular outcomes trial (CVOT) to evaluate the cardiovascular risk in patients with high cardiovascular disease risk and the evaluation of bladder cancer risk in patients enrolled in the CVOT[20].

Although there are several SGLT-2 inhibitors in varying phases of development, canagliflozin and dapagliflozin will be presented here due to availability of human safety and efficacy data.

Canagliflozin (invokana®) clinical trials

Wilding *et al*[14] designed a randomized, double-blind, placebo-controlled, phase 3, multicenter, 52-wk study to evaluate the safety and efficacy of canagliflozin added to metformin plus sulphonylurea in patients with type 2 diabetes.

The trial, called CANagliflozin Treatment And Trial Analysis-Metformin plus SUIphonylurea, included patients if they were 18-80 years with type 2 diabetes, who were stable on maximum or near maximum dosages of metformin and sulfonylureas with an A1c $\geqslant$ 7% and $\leqslant$ 10.5%[14].

The primary efficacy endpoint was A1c change from baseline to 26 wk. The secondary end points included change in baseline A1c at 52-wk, change in baseline in fasting plasma glucose (FPG), systolic blood pressure (BP), percent change in body weight, triglycerides, and high density lipoprotein (HDL) cholesterol, and percent patients reaching A1c 7%[14]. The investigators evaluated safety by observing adverse event reports, vital signs and laboratory tests[14]. Patients were randomized to receive either 100 mg or 300 mg canagliflozin or placebo in addition to their metformin and sulphonylurea therapies[14].

Results of the study show that 381 (81%) of 469 patients, who were randomized to the study, completed the 52-wk study. By week 26, the A1c was significantly reduced in the canagliflozin 100 mg and 300 mg study arm to -0.85% and 1.06% which was statistically significant compared to baseline and the A1c was sustained over the entire 52 wk study period[14]. Results are presented in Table 2[14]. FPG was significantly improved at 26 wk and 52 wk with both canagliflozin 100 mg and 300 mg compared to placebo. Canagliflozin significantly reduced weight but there were no significant changes with systolic blood pressure, pulse or cholesterol parameters[14].

Safety profile and adverse events: Although investigators reported that adverse effects were higher with canagliflozin than placebo, they were comparable across the treatment groups. Patients on canagliflozin had higher rates of genital mycotic infections compared to placebo, which were described as mild to moderate in severity[14]. Patients who developed a mycotic infection, especially women, had a prior history of genital mycotic infections compared to those women who received canagliflozin and did not have adverse effects[14]. Genital mycotic infections were treated without interrupting canagliflozin therapy[14].

Canagliflozin compared to sitagliptin

Canagliflozin has been shown to be non-inferior to sitagliptin and in another analysis superior to sitagliptin with regard to lowering of A1c[16].

In a randomized, double-blind, active-control, multicenter, phase three, 52-wk study, Schernthaner evaluated the efficacy and safety of canagliflozin 300 mg compared with sitagliptin 100 mg as add-on therapy in patients with type 2 diabetes mellitus inadequately controlled with metformin and a sulfonylurea[16].

The inclusion criteria were similar to the previously described study, and patients were randomized to receive either 300 mg canagliflozin or 100 mg sitagliptin[16]. The primary efficacy endpoint was A1c change from baseline to 52 wk while the secondary endpoints were similar to the previously described study[16].

Results of the study show that 464 (61%) of 755 patients, who were randomized to receive either canagliflozin 300 mg or sitagliptin 100 mg daily, completed the study. Most of the withdrawals were observed in the sitagliptin therapy arm of the trial due to the lack of glycemic rescue therapy[16]. Canagliflozin demonstrated both noninferiority and in another analysis, showed superiority to sitagliptin 100 mg in reducing A1c (-1.03% and -0.66%, respectively). There were greater reductions with canagliflozin *vs* sitagliptin in FPG, body weight, and systolic BP. More patients on canagliflozin compared with sitagliptin achieved A1c < 7.0%, and A1c < 6.5% at week 52, though the authors did not confirm statistical significance[16]. Results are presented in Table 3[16].

Table 2 Results of phase 3, CANagliflozin treatment and trial analysis-metformin plus SUlphonylurea, *n* = 469[14]

Parameters	Canagliflozin 100 mg	Canagliflozin 300 mg	Placebo	Comments
A1c (%) week 26	-0.85	-1.06	-0.13	$P < 0.001$
A1c (%) week 52	-0.74	-0.96	-0.01	$P < 0.001$
% Patients with A1c < 7% week 26	43.2	56.6	18.0	$P < 0.001$
% Patients with A1c < 7% week 52	39.4	52.6	18.7	$P < 0.001$
FPG (mg/dL) week 26	-21.6	-34.2	-	$P < 0.001$
FPG (mg/dL) week 52	-28.8	-37.8	-	$P < 0.001$
Weight	-1.10	-1.7	-	$P < 0.001$
Change in systolic blood pressure (mmHg)	-2.20	-1.6	-	Non significant
Change in pulse (beats/min)	0.90	-1.2	-0.4	Non significant

A1c: Hemoglobin A1c; FPG: Fasting plasma glucose.

Table 3 Results of canagliflozin compared with sitagliptin for patients with type 2 diabetes: (*n* = 755)[16]

Parameters	Canagliflozin 300 mg	Sitagliptin 100 mg	Comments
A1c (%) week 52	-1.03	-0.66	Non inferiority to sitagliptin (upper limit of the 95%CI < 0.3%) and superiority to sitagliptin (upper limit of the 95%CI < 0.0%)
Percent (%) of patients with A1c < 7% at week 52	47.6	35.3	Not significant
Percent (%) of patients with A1c < 6.5% at week 52	22.5	18.9	Not significant
FPG (mg/dL) week 26	-29.9	-5.9	$P < 0.001$
Weight (kg)	-2.3	-0.1	$P < 0.001$
Change in systolic blood pressure (mmHg)	-5.1	0.9	$P < 0.001$
Change in diastolic blood pressure (mmHg)	-3.0	-0.3	Not significant

A1c: Hemoglobin A1c; FPG: Fasting plasma glucose.

Safety profile and adverse events: There were no differences in adverse effects, hypoglycemia or discontinuation of therapy between treatment groups. Nevertheless, canagliflozin had higher rates of genital mycotic infections (vulvovaginitis in females and balanitis in males) compared to sitagliptin[16]. In other studies, canagliflozin is implicated in urinary tract infections, hypoglycemia and gastrointestinal upset when used alone or in combination with other antihyperglycemic therapy[21].

Canagliflozin was associated with a dose dependent increase in serum creatinine, decrease in estimated glomerular filtration rate, renal impairment, and acute failure in patients especially those with moderate renal impairment and hypovolemia[22].

Canagliflozin 100-300 mg is recommended for patients with creatinine clearance > 60 mL/min per 1.73 m^2 and canagliflozin 100-mg is recommended for patients with creatinine clearance of 45-60 mL/min per 1.73m^2[22]. Canagliflozin is not recommended in patients with creatinine clearance of 30-44 mL/min per 1.73 m^2, and it is contraindicated in patients with creatinine clearance of < 30 mL/min per 1.73m^2[22]. Clinicians should assess patients' renal functions when initiating therapy and for long term drug monitoring. This agent will be a safe and efficacious addition to a dual therapy regimen such as metformin and sulfonylurea based on this study[16].

DAPAGLIFLOZIN AS MONOTHERAPY

List *et al*[23] designed a prospective, dose ranging 12-wk, randomized parallel-group, double-blind, placebo-controlled study to evaluate the safety and efficacy of dapagliflozin. The primary objective was to compare the mean change from baseline A1c in type 2, treatment-naïve adult patients (age 18-79) with A1c ≥ 7% and ≤ 10%[23].

Patients were randomly assigned to one of five once-daily dapagliflozin doses (2.5, 5, 10, 20 or 50 mg), metformin XR (750 mg force titration to 1500 mg) or placebo. Investigators also evaluated changes in FPG, weight, and adverse effects[23].

Results of the study show that 348 (89%) of 389, who were randomized to the study completed the study at week 12[23]. At the end of the study, dapagliflozin had statistically significant mean dose-dependent reduction of A1c from -0.55% to -0.90% when compared with placebo -0.18% but not with metformin of -0.73%[23]. Dapagliflozin also had significant reduction in FPG of -16 to -31 mg/dL compared to 6 mg/dL with placebo and -18 mg/dL with metformin[23]. Dapagliflozin caused a weight loss change of -1.3 to 2 kg[23]. In this trial, dapagliflozin did not demonstrate any renal function changes[23]. The percentage of patients achieving A1c < 7% was 40%-59% for the dapagliflozin group *vs* 32% for placebo and 54% for metformin[23]. Hypoglycemia was reported in 6%-10% of patients treated with dapagliflozin but this was not dose related, compared to 4% of placebo patients and 9% of metformin-treated patients[23].

Dapagliflozin in combination with metformin

Henry *et al*[24] conducted two randomized, double-blind,

Table 4 Dapagliflozin in combination with metformin[24]

Parameters	Study 1			Study 2		
	DAPA 5 ± MET	DAPA 5 ± PBO	MET ± PBO	DAPA 10 ± MET	DAPA 10 ± PBO	MET ± PBO
A1c at 24 wk (%)						
Baseline (*n*)	9.21 (185)	9.14 (196)	9.14 (195)	9.1 (202)	9.03 (216)	9.03 (203)
A1c (%) at 24 wk (baseline change)	7.13 (-2.05)	7.96 (-1.19)	7.79 (-1.35)	7.1 (-1.98)	7.59 (-1.45)	7.6 (-1.44)
DAPA ± MET *vs* DAPA	-0.86 (-1.11, -0.62)			-0.53 (-0.74, -0.32)		
P value	< 0.0001			< 0.0001		
DAPA ± MET *vs* MET	-0.70 (-0.94, -0.45)			-0.43 (-0.75, -0.33)		
P value	< 0.0001			< 0.0001		
Patients with A1c < 7% at 24 wk						
n (%)	96/185 (52.4%)	46/196 (22.5%)	68/195 (34.6%)	92/202 (46.6%)	69/216 (31.7%)	72/203 (35.2%)
DAPA ± MET *vs* DAPA	29.9	22.5		14.9		
P value	< 0.0001			0.0012		
DAPA ± MET *vs* MET	17.8			11.3		
P value	< 0.0001			0.0165		
Plasma glucose at 24 wk (mg/dL)						
Baseline FPG (mg/dL)	193.14 (*n* = 192)	190.62 (*n* = 203)	196.56 (*n* = 200)	189.36 (*n* = 209)	197.28 (*n* = 216)	189.72 (*n* = 207)
FPG after 24 wk (baseline change)	132.3 (-61.02)	150.3 (-41.94)	161.1 (-33.48)	130.86 (-60.3)	147.6 (-46.44)	156.42 (-34.74)
DAPA ± MET *vs* DAPA	-19.08			-13.86		
P value	< 0.0001			< 0.0001		
DAPA ± MET *vs* MET	-27.54			-25.56		
P value	< 0.0001			< 0.0001		
Total body weight at 24 wk (kg)						
Baseline weight (*n*)	84.24 (192)	86.20 (203)	85.75 (200)	88.56 (209)	88.53 (219)	87.24 (208)
Change from baseline	-2.66 (-3.14, -2.19)	-2.61 (-3.07, -2.15)	-1.29 (-1.76, -0.82)	-3.33 (-3.80, -2.86)	-2.73 (-3.19, -2.27)	-1.36 (-1.83, -0.89)

DAPA: Dapagliflozin; MET: Metformin; PBO: Placebo; FPG: Fasting plasma glucose; A1c: Hemoglobin A1c.

three-arm 24-wk trials to compare the combination of dapagliflozin plus metformin *vs* dapagliflozin monotherapy and metformin monotherapy to determine if the combination would be an advantage for treatment naïve type 2 diabetes patients with high baseline A1c.

Study 1 compared dapagliflozin 5 mg in combination with metformin XR, dapagliflozin 5 mg in combination with placebo, and metformin XR plus placebo. Study 2 compared dapagliflozin 10 mg in combination with metformin XR, dapagliflozin 10 mg in combination with placebo, and metformin XR plus placebo[24].

Eligible patients had a baseline A1c 7.5%-12%, and the primary endpoint was a change in A1c from baseline while the investigators also evaluated the change in FPG and weight as secondary endpoints[24].

Results show that in both trials, the combination of dapagliflozin and metformin resulted in significantly lower reductions in A1c compared with either metformin or dapagliflozin monotherapy[24]. Results of the study are presented in Table 4[24]. The combination therapy was statistically superior to monotherapy in reduction of FPG and was more effective than metformin for weight reduction. Dapagliflozin 10 mg was non-inferior to metformin in reducing A1c in study 2[24].

Safety profile and adverse events: Adverse effects of mild to moderate cases of genital infection of vulvovaginitis and balanitis and urinary tract infections were reported and treated without discontinuing the study[24]. There were no major hypoglycemic events reported. Diarrhea was more common in patients on combination therapy with metformin than with dapagliflozin therapy alone[24].

Summary of SGLT-2 inhibitors: Canagliflozin and dapagliflozin have been shown to lower renal threshold for glucose in a dose dependent fashion by increasing urinary glucose excretion through SGLT-2 inhibition, which leads to clinical significant reduction in A1c, FPG, and body weight[14,24]. The reduction in renal threshold is above the threshold for hypoglycemia demonstrating this agent has a low risk of hypoglycemia[17]. The SGLT-2 inhibitors can be used with any other agent whether in a treatment naïve patient or a patient with a long history of type 2 diabetes[22,23,25]. Both therapies are safe and tolerable, but clinicians need to observe for genital infections, which can be easily treated without discontinuation of therapy.

METABOLIC APPROACHES TO THERAPY

11β-HSD-1 inhibitors

High levels of glucocorticoids have been associated with hyperglycemia, insulin resistance, dyslipidemia and visceral obesity[4]. 11β-HSD is an enzyme, presenting as two distinct isoenzymes: 11β-HSD-1 and 11β-HSD-2. 11β-HSD-1 is found in the liver and adipose tissue and converts inactive cortisone to active cortisol while 11β-HSD-2 is found primarily in the kidneys and colon and it inactivates glucocorticoids by converting active cortisol to inactive cortisone[4,26].

It has been suggested that the increased glucocorticoid activity in the white adipose tissue by 11β-HSD-1 is a key player in the development of visceral obesity, insu-

Table 5 Efficacy assessment of INCB13739 in combination with metformin[30]

	Placebo	5 mg	15 mg	50 mg	100 mg	200 mg
Baseline A1c (%)	8.3 ± 1	8.2 ± 1	8.3 ± 1	8.3 ± 1	8.2 ± 1	8.2 ± 1
LS mean change A1c (%) from baseline	0.09 ± 1	-0.21 ± 1[b,e]	-0.11 ± 1	-0.09 ± 2	-0.38 ± 1[a,e]	0.47 ± 1[d,h]
A1c > 8% (*n*)	-0.10 ± 0.2 (23)	-0.39 ± 0.2[e] (23)	-0.24 ± 0.2 (18)	-0.65 ± 0.3[b,e] (11)	-0.72 ± 0.2[a,e] (16)	0.65 ± 0.2 (19)
A1c (%) for BMI > 30 mg/m^2 (*n*)	0.17 ± 0.1 (29)	-0.24 ± 0.2[b,f] (23)	-0.10 ± 0.2 (26)	-0.25 ± 0.2[b] (18)	-0.36 ± 0.2[a] (26)	-0.76 ± 0.2[d,h] (18)
Baseline FPG (mg/dL)	179 ± 51	172 ± 41	175 ± 44	178 ± 53	170 ± 64	165 ± 41
LS mean change from baseline (mg/dL)	12.6 ± 6.1	6 ± 6.3	2.3 ± 6.4	-4.7 ± 7.2[b]	-1.6 ± 6.1[b]	-11.5 ± 6.2[d,f]
Weight (kg)	-0.2 ± 0.3	-0.5 ± 0.38	-0.6 ± 0.4[e]	0 ± 0.4	-1.1 ± 0.3[b,e]	-0.9 ± 0.3[h]
HOMA-IR	0.25 ± 0.4	-0.29 ± 0.4	0.33 ± 0.4	-0.42 ± 0.5	0.51 ± 0.4	-1.06 ± 0.4[a,e]

Data are placebo adjusted least-squares (LS) mean change from baseline: mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.01$, active *vs* Placebo, [e]$P < 0.05$, [f]$P < 0.01$, [h]$P < 0.01$, week 12 *vs* baseline. A1c: Hemoglobin A1c; FPG: Fasting plasma glucose; LS: Least squares; BMI: Body mass index; HOMA-IR: Homeostatic model assessment-insulin resistance.

lin resistance, diabetes, type 2 diabetes, dyslipidemia and hypertension in mice[27]. Increased levels of 11β-HSD-1 in adipose tissue produce a metabolic syndrome in mice while 11β-HSD-1 deficiency or inhibition has beneficial metabolic effects on liver metabolism[27].

In humans, researchers discover that though patients with glucocorticoid excess develop central obesity, yet the circulating glucocorticoid levels are normal. The metabolic syndrome resembles Cushing's syndrome, but without the elevated circulating glucocorticoid levels. Researchers suggest that it is the increased activity of 11β-HSD-1 in humans, which is metabolizing cortisol from cortisone within adipose tissue that may play a major role in the pathophysiology of obesity[28]. Inhibition of this enzyme may potentially decrease weight and blood glucose.

Non selective 11β-HSD-1 inhibitors

Older non-selective 11β-HSD-1 inhibitors such as liquorice and its active metabolite glycyrrhizic and glycyrrhetinic acids inhibit both 11β-HSD-1 and 11β-HSD-2 enzymes[29].

Ingesting liquorice and glycyrrhizic or glycyrrhetinic acids have been shown to produce a type of "mineralocorticoid excess" syndrome, hypertension encephalopathy, and hypokalemic paralysis[29]. It can also cause weight loss, sodium retention, potassium loss, and hypertension through the inhibition of 11β-HSD-2[29].

Carbenoxolone, a non-selective 11β-HSD-1 inhibitor and product of liquorice reduces glucose concentrations and increases weight loss; inhibits hepatic triglyceride production, inhibits lipolysis, and increase HDL-C levels, but also causes sodium retention, potassium loss, and hypertension by inhibiting 11β-HSD-2[29].

Vitamin A enriched diets also decrease fat and improve insulin sensitivity in animals and humans as it may inhibit 11β-HSD-1 and mRNA[29]. These non-selective agents were evaluated in small trials with short durations[29].

Several 11β-HSD-1 inhibitors have been developed and are being tested for patients with obesity and diabetes, including INCB013739, MK0916, PF915275, AMG221 produced by a variety of manufacturers. Results from INCB013739 clinical studies show that 11β-HSD-1 inhibitors when administered to patients with type 2 diabetes for 2 wk prevented the conversion of oral cortisone to cortisol, decreased hepatic gluconeogenesis, decreased fasting plasma glucose and low density lipoprotein cholesterol[30].

Clinical trial of INCB13739 (a 11β-HSD-1 inhibitor)

Rosenstock *et al*[30] evaluated the efficacy and safety of the agent INCB13739 (an 11β-HSD-1 inhibitor) for patients with type 2 diabetes, who were inadequately controlled on a mean dosage of 1.5 g daily of metformin therapy.

The study was a double-blind, placebo-controlled parallel study conducted with 302 type 2 diabetes mellitus patients on metformin therapy with an A1c of 7% to 11%[30]. Patients received one of five dosages (5, 15, 50, 100 or 200 mg) of INCB13739 or placebo once daily for 12 wk in addition to metformin. The primary end point was a change in A1c at the end of 12 wk. Investigators also reviewed FPG, lipids, weight loss, and adverse events[16,30]. Patients had a mean duration of type 2 diabetes of 6.2 years with baseline body mass index of 32.4 kg/m^2, A1c 8.3% and FPG 173 mg/dL[30].

Results of the study show that 228 of 302 (75%) patients completed the study[30]. At the end of the study, INCB13739 resulted in a dose dependent reduction in A1c of -0.38% and -0.47% in the 100 mg and 200 mg groups respectively[30]. However, it was noted that there were more significant A1c changes in obese patients on the higher dosages[30]. In addition, those with A1c > 8% had more significant decrease in A1c which was dosage dependent[30]. Results of the study are presented in Table 5[30]. The investigators reported that at the end of 12 wk, 25% of patients who were randomized to the 100 mg and 200 mg therapy groups achieved an A1c < 7% compared to 9.5% of placebo patients[30]. FPG decreased in a dose and time dependent fashion in the 100-200 mg treatment groups while there was significant weight loss in the 15, 100 and 200 mg groups[30]. The investigators reported that this study group had generally controlled blood pressure and plasma lipids at baseline but there was a modest dose dependent decrease in total cholesterol -7 mg/dL (P_{trend} = 0.026) from baseline in the 200 mg group[30]. There was no significant difference with HDL cholesterol[30].

Safety profile and adverse events: The therapy was well

tolerated and adverse events were similar across all treatment groups[30]. There were no serious events reported except for cardiac arrest unrelated to study therapy and there were no hypoglycemia reported. The most common adverse event in four patients was nausea in the 200 mg group but this resolved during continuation of therapy[30].

It was noted that there was also a dose dependent statistically significant reduction in Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) suggesting an insulin sensitizing mechanism of action in the 200 mg group[30]. The authors concluded that in patients with type 2 diabetes inadequately controlled with metformin, INCB13739 added to metformin significantly improved A1C, FPG and HOMA-IR[30]. INCB13739 also decreased weight though it did not affect the waist to hip ratio[30].

Summary: 11β-HSD-1 is increased in the adipose tissues of obese patients and those with the metabolic syndrome. 11β-HSD-1 inhibitors may be a viable option for these patients since it converts inactive cortisol to active cortisol in target tissues, which inhibits pancreatic beta cell insulin production, and prevents peripheral glucose uptake promoting weight loss, and decrease in blood glucose[30]. Researchers and clinicians have questions with regard to effects on the immune system, duration and timing of therapy, the long term effects of weight and lipids, glycemic control, insulin action, atherosclerotic plaque formation and cardiovascular risk[30]. The reduction in A1c was moderate but further studies will answer many of these questions to determine the safety and efficacy of 11β-HSD-1 inhibitors.

Glycogen phosphorylase inhibitors

The liver contributes to glucose production by both gluconeogenesis (glucose synthesis) and glycogenolysis (glycogen breakdown)[31]. Type 2 diabetes is characterized by excessive glucose production and inadequate suppression of hepatic gluconeogenesis postprandially[31].

Except for metformin, the production of gluconeogenesis inhibitors has yielded disappointing results with an increase in compensatory hepatic glycogenolysis, which maintains excessive hepatic glucose production[31,32]. Researchers hypothesized that glycogenolysis inhibition can improve blood glucose control by observing patients with hepatic glycogen storage disease experience intermittent hypoglycemia[31]. Glycogen phosphorylase is an enzyme that catalyzes the breakdown of glycogen to glucose-1-phosphate in the liver and tissues that demand high energy[33].

Hepatic glycogenolysis has a major role in the regulation of plasma glucose levels in diabetic mice, and suggests that glycogen phosphorylase inhibitors may be useful in the treatment of type 2 diabetes[31]. Further studies will elucidate if this is so.

Two types of glycogen phosphorylase inhibitors exist[31]. One is a glucose analog, which binds near the active site of the enzyme, and the other is caffeine and other heteroaromatic analogs which bind at the purine inhibitory site (I-site). The I-site is a target for therapy as compounds which bind at this inhibitory site are more potent in the presence of high glucose concentrations[31]. Researchers hypothesized that the inhibitory activity can be regulated by blood glucose concentrations and the inhibitory activity can decrease as normal blood glucose is achieved, which would decrease the risk of hypoglycemia[31].

CP-91149-a glycogen phosphorylase inhibitor in animal studies: CP-91149 was identified as a potent inhibitor of hepatic glucose production in *in vivo* studies in diabetic ob/ob mice[31]. CP-91149 exhibited rapid dose dependent decreases in plasma glucose concentrations (36-120 mg/dL) at 10, 25, and 50 mg/kg doses ($P < 0.001$) without producing hypoglycemia. Hypoglycemia was defined as glucose < 60 mg/dL for CP91149 in this study[31]. Administration of CP-91149 to normoglycemia non diabetic mice at 25-100 mg/dL did not affect glucose lowering. The glucose lowering of CP91149 was accompanied by an inhibition of hepatic glycogen breakdown in the diabetic ob/ob mice[31].

CP-316819-a glycogen phosphorylase inhibitor: CP-316819 is an analogue of CP-91149, which binds to the inhibitor site of glycogen phosphorylase to prevent its transformation to a more active form of the enzyme[33].

One of the concerns was that this analogue does not demonstrate hepatic specificity, so potentially affecting skeletal tissues and having possible deleterious effects to patients who exercise[33]. In a study by Baker, CP-316819 reduced glycogen phosphorylation activation in rat skeletal muscle at rest and maximal contraction, which produced a modest reduction in muscle lactate production[33]. According to the researcher, the study demonstrated that the concern related to potential negative effects of glycogen phosphorylase inhibition on quality of life due to impaired muscle function are unfounded[33].

Summary of glycogen phosphorylase inhibitors

These findings support the possible use of the glycogen phosphorylase inhibitors as a possible addition to the treatment of patients with type 2 diabetes. Further studies are needed to evaluate the effects of glycogen phosphorylase inhibition after chronic oral dosages and under a variety of exercise activities[33].

PROTEIN TYROSINE PHOSPHATASE 1B INHIBITORS

Type 2 diabetes and obesity are both characterized by insulin and leptin resistance[34,35].

Insulin resistance is found in tissues important for glucose homeostasis such as the liver, fat, central nervous system and muscle[34]. Leptin suppresses food intake and increases energy expenditure, but its levels are elevated in obesity demonstrating leptin resistance. Protein tyrosine phosphatases play a major role in leptin resistance by suppressing leptin signaling[36].

Protein tyrosine phosphatase 1B (PTP-1B) is an enzyme that removes phosphate from tyrosine residues in protein such as insulin receptors, so it is described as a negative regulator for insulin and leptin, by dephosphorylating phosphorylated tyrosine residues from the insulin receptor[34]. PTP-1B activity is increased in insulin resistance and obese patients[34].

Summary

Diabetes mice treat with specific PTP-1B inhibitors exhibited normalized BG control, improved insulin sensitivity, and modulated fat storage, and lipogenesis in adipose tissue[34]. Therefore these inhibitors have emerged as a potential oral agent that can provide a strategy for the treatment of type 2 diabetes and obesity and may work best in patients with beta cell function that releases insulin[35].

Further studies will elucidate if these agents can also be a potential addition to the armamentarium of oral diabetes agents affecting both obesity and the metabolic syndrome.

G-PROTEIN-COUPLED RECEPTOR 119 AGONISTS

A dysfunction in pancreatic β cell leading to decreased insulin secretion is a major abnormality in type 2 diabetes mellitus[37]. The pharmacotherapy approach of stimulating insulin release in a glucose-dependent manner using G-protein-coupled receptor has been investigated[38]. Specifically, G-protein-coupled receptor 119 (GPR119) is largely distributed in pancreatic islet cells, somewhat in the gastrointestinal tract, and found to be involved in glucose metabolism[39-41].

GPR119 may be stimulated by endogenous ligands or synthetic compounds resulting in an elevated cyclic adenosine monophosphate[42]. Studies have shown that stimulation of GPR119 yields glucose-dependent insulin release from the pancreatic β cells, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide secretions from intestinal cells[42]. Thus, pharmacological agents that target GPR119 results in glucose reduction with low hypoglycemia risk, body weight loss, and potential for pancreatic β cell preservation[42]. These characteristics are very similar to the commercially available GLP-1 agonists, however the studied GPR119 agents may be orally administered. Several GPR119 molecules (GSK1292263, MBX-2982, PSN-821, AR231453, AR-7947) have been studied in preclinical and/or early clinical trials with poor outcomes due to loss of pharmacological effect or minimal glycemic lowering effect[42]. Furthermore, GPR119 agonists have also been considered in combination with DPP-4 inhibitors in an attempt to enhance the GLP-1 effects[42].

Summary

GPR119 agonists have strong potential to meet the needs of patients with type 2 diabetes because of their relative safety profile, lack of weight gain, oral formulation, and possible β cell preservation effect. However, there have been challenges to their development due to potential tachyphylaxis and low anti-hyperglycemia efficacy.

GK ACTIVATORS

GK is a key enzyme in the hexokinase family that facilitates glucose homeostasis *via* glucose phosphorylation and metabolism mainly in the pancreatic β cells and hepatocytes[43-45]. GK functions as a glucose sensor in pancreatic β cells, thereby stimulating glucose-stimulated insulin secretion and regulating glucose metabolism within the liver, including gluconeogenesis, glycolysis, glycogen synthesis, glucose oxidation, lipogenesis, urea, and uric acid production[43,45-48].

Since the initial development of small molecules known as GK activators (GKAs) that bind to an allosteric site of the enzyme in 2003, more than 150 patents have been established[49-51]. Preclinical and clinical phase trials of GKAs have demonstrated glucose lowering effect in both animal and humans[52]. This novel class of anti-diabetic agents holds promise particularly because both mechanistic actions of GK are impaired in type 2 diabetes[53]. However, there are concerns about potential side effects including hyperlipidemia, hypoglycemia, and fatty liver that may limit the development of GKAs[54]. For example, a small Phase I clinical trial involving the GKA piragliatin was discontinued in type 2 diabetes patients with unrevealed rationale[55].

Another GKA molecule, MK0941 was evaluated in a 54-wk Phase Ⅱ trial in type 2 diabetes patients, but was discontinued because of observed hyperlipidemia, vascular hypertension and early therapy failure[56].

Summary of GKA

GKAs offer a unique pharmacotherapeutics approach to type 2 diabetes management and have demonstrated useful potential in glycemic management. However, further development is needed to address the potential side effects observed in clinical trials. Additional advancements may include modifications of the GKAs structures and activities to minimize hypoglycemia, hyperlipidemia, fatty liver, and vascular hypertension[44].

CONCLUSION

The management of type 2 diabetes present many treatment challenges, but new and emerging drug therapies are a welcome addition to complement the current agents. The SGLT-2 inhibitors have shown significant benefits as monotherapy and in combination with available agents like metformin, sulphonylurea and insulin therapy. The selective 11β-HSD-1 inhibitor is another class of possibly safe and efficacious agent that lowers fasting blood glucose, A1c and weight, although the A1c lowering was modest. The glycogen phosphorylase inhibitors appear to show rapid and safe blood glucose decreases in mice without the risk of hypoglycemia. Hope-

fully similar results translate into human studies. PTP-1B is still in clinical trials and may show significant decrease in weight and glucose levels in insulin and leptin resistant patients. Mice studies show positive results of normalized blood glucose control, improved insulin sensitivity and improvements in lipogenesis. The GPR119 agonists have strong potential for meeting the needs of type 2 diabetes patients because of their safety profile, lack of weight gain and possible beta cell preservation effect. However, the GK inhibitors may have some potential problems as agents so far have been discontinued due to dyslipidemia, vascular hypertension and early therapy failure. Prescribers and pharmacists may have to recognize that these new agents may not be first line agents due to costs, monitoring parameters, modest reductions of A1c, and lack of cardiovascular disease data. Further studies will help to more clearly define these new and emerging anti-hyperglycemia agents' roles in therapy.

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P- Reviewers: Masaki T, Pastromas S **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Canagliflozin-current status in the treatment of type 2 diabetes mellitus with focus on clinical trial data

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Author contributions: All the authors contributed in collection of data on CFZ; Bhatia J and Gamad N were involved in writing the manuscript; Gamad N and Bharti S compiled the table no. 1 (Summary of clinical trials of CFZ) and table no. 2 (Summary of adverse events observed in the CFZ clinical trials) in the manuscript; Arya DS did the final editing of the review article.

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Received: January 22, 2014 Revised: April 3, 2014
Accepted: April 17, 2014
Published online: June 15, 2014

Abstract

Canagliflozin (CFZ) is a member of new class of glucose lowering agents, sodium-glucose co-transporter (SGLT) inhibitors, which got approval by food and drug administration. It has insulin independent action by blocking the transporter protein SGLT2 in the kidneys, resulting in urinary glucose excretion and reduction in blood glucose levels. In clinical trials, CFZ significantly decreased HbA1c level when administered either as monotherapy or as combined therapy with other anti-diabetic drugs. Intriguingly, it showed additional benefits like weight reduction and lowering of blood pressure. The commonly observed side effects were urinary and genital infections. It has exhibited favorable pharmacokinetic and pharmacodynamic profiles even in patients with renal and hepatic damage. Hence, this review purports to outline CFZ as a newer beneficial drug for type 2 diabetes mellitus.

Key words: Type 2 diabetes mellitus; Sodium-glucose co-transporter 2; Canagliflozin; Clinical trial; Safety profile

Core tip: This review article focuses upon the current pharmacokinetic, pharmacodynamic and clinical trial data on the newly introduced sodium-glucose co-transporter 2 inhibitor, canagliflozin, for the treatment of type 2 diabetes mellitus. It also discusses briefly about the safety profile and future prospective of canagliflozin.

Original sources: Bhatia J, Gamad N, Bharti S, Arya DS. Canagliflozin-current status in the treatment of type 2 diabetes mellitus with focus on clinical trial data. *World J Diabetes* 2014; 5(3): 399-406 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i3/399.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i3.399

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by insulin resistance, hyperglycemia and progressive pancreatic β-cell dysfunction. Poorly controlled hyperglycemia leads to irreversible microvascular and macrovascular complications like visual impairment and blindness, kidney failure, peripheral neuropathy, myocardial infarction, stroke and lower limb amputation. In 2012, worldwide > 371 million people suffered from diabetes. Out of which 4.8 million people died due to its complications. This global burden is estimated to increase to 552 million by 2030[1]. This implies that the available drugs for DM are not able to maintain or achieve good glycemic control. Potential adverse events like gastrointestinal disturbances (with biguanides like metformin, α-glucosidase inhibitors like acarbose, glucagon-like peptide-1 agonists like exenatide, amylin agonists like pramlintide), hypoglycemia (with insulin, secretagogues like sulfonylureas and meglitinides), weight gain (with insulin, secretagogues like sulfonylureas and meglitinides, thiazolidinediones like pioglitazone) and risk of cardiovascular disease (with thiazolidinediones like pioglitazone) limit their dosage; and ensuing β-cell failure limits their effectiveness. Current guidelines recommend a target HbA1c value of < 7.0%, with patient-centered

approach allowing some flexibility in terms of the actual target, and treatment with lifestyle changes and drugs for better glycemic control in diabetics. But the target HbA1c is rarely achieved with a single anti-diabetic agent and in only about half of adult patients with diabetes taking combination therapy[2,3]. Hence, there is ongoing hunt for newer efficacious and safer treatment strategies.

Kidney plays a pivotal role in maintaining glucose homeostasis through specialized transporters-sodium-glucose co-transporter (SGLT)1 and SGLT2-present in the proximal convoluted tubule (PCT). Together, they absorb almost all of the glucose filtered in the glomerulus. SGLT1 is a low capacity, high affinity transporter present mostly in small intestine, some in S3 segment of PCT in kidney, and in heart. It is responsible for approximately 10% of glucose reabsorption in the kidney. While SGLT2 is a high capacity, low affinity transporter present almost exclusively in S1 segment of PCT, responsible for approximately 90% of glucose reabsorption[4,5]. But kidney was never the target for treatment of diabetes until phlorizin was discovered. Phlorizin was isolated from the apple trees in 1835 and was initially tested for fever, infectious diseases and malaria. It was noticed that high doses caused glycosuria and chronic administration in dogs caused polydipsia and polyuria with normoglycemia. Subsequent detection of SGLT1 and SGLT2 in kidney, their role in glucose reabsorption and confirmation of inhibitory action of phlorizin on these transporters in animal studies paved way to consider phlorizin in the treatment of type 2 diabetes mellitus (T2DM). However, phlorizin was not clinically developed due to its poor pharmacokinetics and side effects attributed to SGLT1 inhibition such as glucose-galactose malabsorption, dehydration and diarrhea[6,7]. Later on T-1095 was discovered, a derivative of phlorizin which had comparatively better pharmacokinetic profile. Nevertheless, it was discontinued in the Phase-Ⅱ clinical trial[8]. Meanwhile, it was observed that there was upregulation of SGLT2 and increase in maximum tubular transport of glucose in diabetic patients[9]. The underline defect in patients with familial renal glycosuria is also attributable to SGLT2 gene mutation. The patients with gene defect excrete increased amount of glucose in urine and are clinically asymptomatic[10]. These two observations with SGLT2 transporter, *i.e.*, the upregulation of SGLT2 in diabetes and its role in familial renal glycosuria, triggered research that ultimately led to the discovery of specific SGLT2 inhibitors viz. sergliflozin and remogliflozin. Unfortunately, these drugs too exhibited unfavorable pharmacokinetic profile, efficacy and side effect and hence did not progress in clinical trials[11].

Dapagliflozin is the first SGLT2 inhibitor that came to the European market in 2012. Food and drug administration (FDA) approved dapagliflozin on 8th January, 2014[12]. It was initially rejected by FDA due to serious concerns about bladder and breast cancer[13]. Canagliflozin was the first of its kind to get approval from FDA on March 29, 2013. Currently it is in phase-Ⅱ trial for the treatment of obesity in the United States and Europe[14]. Ipragliflozin, empagliflozin and many other SGLT2 inhibitors are under different phases of clinical trials.

This article reviews the available data on the pharmacokinetics, the pharmacodynamics and the therapeutic potential and safety of canagliflozin (CFZ).

SEARCH METHODOLOGY

PubMed, ClinicalTrials.gov and Google scholar databases were used for mining the data. Following Medical subject headings words were used in the above mentioned databases: canagliflozin, canagliflozin and SGLT2, canagliflozin and diabetes, canagliflozin and pharmacokinetics, canagliflozin and pharmacodynamics and canagliflozin and adverse events. Up to date information was included till 31st March 2014.

PHARMACOKINETIC PROPERTIES

When CFZ is taken orally it gets rapidly absorbed from gastrointestinal tract in a dose dependent manner with the dose range of 50-300 mg and mean oral bioavailability of approximately 65%. Median $t_{1/2}$ is 1-2 h and steady state concentration is achieved after 4 to 5 d of daily intake of 100 mg and 300 mg. Maximum plasma concentration is not altered in renal injury. It accumulates in the plasma up to 36% following multiple doses of 100 and 300 mg. The plasma protein binding is 99%, which is constant irrespective of its plasma concentrations or hepatic or renal damage[15,16]. It is metabolized into two inactive O-glucuronide metabolites (M5 and M7). Major O-glucuronidation is by UDP glucuronosyltransferase (UGT)1A9 and UGT2B4, while CYP3A4 mediated oxidative metabolism accounts for only 7%. Single oral radioactive [^{14}C] CFZ to healthy subjects demonstrated 41.5%, 7.0% and 3.2% of administered radioactive dose in feces as CFZ, a hydroxylated metabolite and an O-glucuronide metabolite, respectively. The amount of CFZ excreted in urine in unchanged form is less than 1%, whereas the urine excretion of its metabolites namely M7 is 21%-32% and M5 is 7%-10%. Studies conducted so far have shown no clinically significant effect of age, sex, BMI/weight and race on pharmacokinetics of CFZ[15,16].

PHARMACODYNAMIC PROPERTIES

CFZ primarily inhibits SGLT2 in kidney and is responsible for increased urinary glucose excretion and reduction in blood glucose levels. It also inhibits SGLT1 in intestine and its potency on SGLT1 is 160 times lesser as compared to SGLT2[15,16]. It reduces glucose absorption by 31% in first hour and 20% by next hour of food intake. So, when given before meal, it reduces postprandial glucose excursions[15,17]. This insulin independent action is unique and differentiates CFZ from other available antidiabetic agents. Moreover, there is dose dependent reduction in the renal threshold for glucose excretion (RT_G)

with maximal suppression of RTG from 240 mg/dL to approximately 70-90 mg/dL at the dose of 300 mg. Unlike other oral hypoglycemic drugs, CFZ is tolerated well in mild to moderate hepatic and renal failure patients. However, it is contraindicated in patients with estimated GFR (eGFR) < 30 mL/min per 1.73 m^2, end stage kidney disease and patients on dialysis[15].

DOSAGE AND ADMINISTRATION

The recommended starting dose of CFZ is 100 mg once daily to be taken before the first meal of the day. If patients with eGFR of ⩾ 60 mL/min per 1.73 m^2 tolerate CFZ 100 mg once daily and require additional glycemic control, then dose can be increased to 300 mg once daily. Volume depletion has to be corrected in patients prior to the initiation of CFZ to compensate for CFZ induced increased urination[15].

DRUG INTERACTIONS

UGT inducers (*e.g.*, rifampin, phenytoin, phenobarbital, ritonavir) increase the metabolism of CFZ, thereby reducing active CFZ levels in the blood. Thus, the dose of CFZ may be increased from 100 to 300 mg in such patients. On the other hand, CFZ increases Area Under the Curve for digoxin and hence patients on digoxin treatment should be monitored[15].

THERAPEUTIC POTENTIAL

CFZ has shown promising results in many preclinical and clinical studies of T2DM. A study in Zucker fatty rats and Zucker diabetic fatty rats with CFZ (3-30 mg/kg) decreased renal threshold for glucose and increased urinary glucose excretion (UGE). This resulted in decreased blood glucose, HbA1c, weight gain, dose dependent increased fatty acid metabolism, *de novo* lipogenesis and improved insulin sensitivity in these animals[18].

Table 1 lists the published clinical trials on CFZ use as monotherapy and combined therapy. The CANagliflozin Treatment And Trial Analysis (CANTATA Trials) evaluated CFZ as monotherapy or as an add-on therapy to metformin, metformin and sulphonylurea and metformin and pioglitazone. These trials were randomized; double blind, placebo-or active-controlled with primary endpoint of finding the change in HbA1c at the end of 26 or 52 wk from baseline. In a trial using CFZ as monotherapy, both the doses 100 mg and 300 mg produced a statistically significant decrease in HbA1c ($P < 0.001$), body weight (-2.8% by 100 mg and -3.9% by 300 mg *vs* placebo, $P < 0.001$) as well as systolic blood pressure (-3.7 mmHg by 100 mg and -5.4 mmHg by 300 mg *vs* placebo, $P < 0.001$)[19]. Similar significant results were obtained in combined therapy trials viz. CANTATA-D (Dual therapy trial-CFZ compared with Sitagliptin)[20] and CANTATA-MP (CFZ compared with metformin and pioglitazone)[21].

The CANTATA-SU (CFZ compared with Sulphonylurea) trial established reductions in HbA1c in the glimepiride and CFZ 100 mg groups but greater reductions occurred in CFZ 300 mg group. CFZ 100 mg was reviewed as non-inferior where as CFZ 300 mg group was considered as superior to glimepiride arm. There was greater reduction in body weight, blood pressure (BP) and greater rise in high density lipoprotein (HDL) levels in CFZ group[23]. CANTATA-MSU (CFZ compared with metformin and sulphonylurea) results also demonstrated statistically significant reductions ($P < 0.001$) in HbA1c, fasting blood glucose (FBG) and body weight[24]. In another CANTATA-D2 (Triple therapy trial-CFZ compared with Sitagliptin) trial, at the end of 52 wk, it was showed that CFZ 300 mg was superior to sitagliptin 100 mg when added to sulphonylurea and metformin, in reducing HbA1c, FBG, body weight and systolic blood pressure. There was also significant increase in HDL ($P < 0.001$) in CFZ groups as compared to sitagliptin 100 mg[25].

CANTATA trials have unveiled various interesting clinical observations of CFZ use in the management of T2DM patients. CFZ improved glycemic control without a concomitant increase in the occurrence of hypoglycemia. It lowered RTG but lowering of RTG remained above the hypoglycemic threshold (60-70 mg/dL) and since UGE occurs below the RTG, the incidence as well as risk of hypoglycemia with CFZ was minimal[19,26]. Further, the amplified UGE of 80-120 g/d accounted for net loss of calories (approximately 400 kcal/d) that contributed to the weight loss, which was maintained over the trial period of 52 wk[24,26]. This weight loss was predominantly from loss of fat mass rather than lean body mass[22]. The reversal of glucotoxicity and weight loss together helped to improve beta cell function as indicated in improvement in Homeostasis Model Assessment estimating steady state beta cell function in percentage[19,21,24,26]. The mechanism for increased low-density lipoprotein-C with CFZ is not known, however, improvement in HDL-C and triglycerides was likely to be due to improved glycemic control and weight loss associated with CFZ[19,21,22]. Mild reduction in BP was also observed in the trial participants. This was due to the mild osmotic diuretic response to UGE and natriuretic effect of CFZ[24]. Thus, in nutshell, CFZ can reduce blood glucose levels and has the least risk of producing hypoglycemia as compared to other anti-diabetic agents. In addition, it can also modify the insulin resistance, reduce weight and BP and increase HDL-C. These diverse effects are specific to CFZ and would explain the better outcome with CFZ treated patients as compared to other anti-diabetic agent treatment groups. The CANTATA trials have concluded that CFZ could be taken as an initial drug for T2DM patients whose glycemic control is not achieved with diet and exercise; and also as an effective alternative to sulphonylurea, sitagliptin or pioglitazone in dual therapy with metformin.

CFZ was also studied as an add-on to insulin therapy in a 28-d trial. Participants were T2DM patients not optimally controlled with insulin and receiving up to one oral

Table 1 Summary of clinical trials of canagliflozin

References/trial name (*n* = sample size)	Study population and duration of the study	Study drugs	Change in HbA1c from the baseline (in percent)	Change in FBG from baseline	Change in body weight from baseline	Other parameters (least square mean change)
Stenlöf *et al*[19] (*n* = 584)	T2DM patients on diet and exercise with inadequate glycemic control Duration of the study = 26 wk	CFZ = 100 mg/300 mg OD *vs* PL	-0.77% to -1.03% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	-27 mg/dL to -35 mg/dL (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	% Body weight reduction -2.8% to -3.9% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	ΔPPBG = -43 to -59 mg/dL (CFZ 100 mg/300 mg) ΔSBP = -3.3 to -5.0 mmHg (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL) ΔDBP = -1.7 to -2.1 mmHg (CFZ 100 mg/300 mg *vs* PL) ΔHDL = CFZ 100 mg = 11.2% ($P < 0.001$ *vs* PL) CFZ 300 mg = 10.6% ($P < 0.01$ *vs* PL) ΔLDL = 2.9% to 7.1% (CFZ 100 mg/300 mg *vs* PL) ΔTG = 2.5% to -2.3% (CFZ 100 mg/300 mg *vs* PL) HOMA-%B = 9.9% to 20.3% (CFZ 100 mg/300 mg *vs* PL)
CANTATA-D[20] (*n* = 1284)	26-wk extension study T2DM patients with inadequate glycemic control on protocol specified MET-IR monotherapy with HbA1c: 7.0% to 10.5%, FBG < 270 mg/dL	CFZ = 100 mg/300 mg OD + MET-IR *vs* PL + MET-IR for first 26 wk	At the end of 26 wk -0.79% to -0.94% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk -27.3 mg/dL to -37.8 mg/dL (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk % Body weight reduction -3.7% to -4.2% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk ΔPPBG = -47.9 mg/dL to -57.1 mg/dL (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL) SITA = -49.3 mg/dL ΔSBP = -3.84 mmHg to -5.06 mmHg (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL), SITA = -1.83 mmHg ΔTG = CFZ 100 mg = 1.6%, $P = 0.7$ *vs* PL, CFZ 300 mg = -1.4%, $P = 0.2$ *vs* PL, SITA = 1.0%
		CFZ = 100 mg/300 mg OD + MET-IR *vs* SITA 100 mg + MET-IR for next 26 wk	SITA = -0.82%	SITA = -20.2 mg/dL	SITA = -1.2%	ΔHDL = 10.4% to 12.1% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL), SITA = 5%
			At the end of 52 wk -0.73% to -0.88% (CFZ 100 mg/300 mg)	At the end of 52 wk -26.2 mg/dL to -35.2 mg/dL (CFZ 100 mg/300 mg, $P < 0.001$ *vs* SITA)	At the end of 52 wk -3.8% to -4.2% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* SITA)	At the end of 52 wk ΔSBP = -3.53 mmHg to -4.65 mmHg (CFZ 100 mg/300 mg, $P < 0.001$ *vs* SITA) SITA = -0.66 mmHg
			SITA = -0.73%	SITA = -17.7 mg/dL	SITA = -1.3%	ΔTG = CFZ 100 mg = 1.9%, $P = 0.46$ *vs* SITA CFZ 300 mg = 2.7%, $P = 0.32$ *vs* SITA SITA = -0.4% ΔHDL = 11.2% to 13.3% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* SITA), SITA = 6.0%
Guthrie *et al*[21] Forst *et al*[22] CANTATA-MP (*n* = 344)	26-wk extension study T2DM patients currently treated with PPAR gamma agent (PIO or ROSI) and MET with HbA1c: 7%-10.5% and FBG < 270 mg/dL	CFZ = 100 mg/300 mg OD + MET + PIO *vs* PL + MET + PIO for first 26 wk	At the end of 26 wk -0.89% to -1.03% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk -26.8 mg/dL to -33.2 mg/dL (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk -2.8% to -3.8% (CFZ 100 mg/300 mg, $P < 0.001$ *vs* PL)	At the end of 26 wk ΔSBP = CFZ 100 mg = -5.30 mmHg ($P = 0.005$ *vs* PL) CFZ 300 mg = -4.70 mmHg ($P = 0.016$ *vs* PL) ΔTG = CFZ 100 mg = 3.2% ($P = 0.034$ *vs* PL) CFZ 300 mg = -1.7% ($P = 0.003$ *vs* PL) ΔHDL = CFZ 100 mg = 7.2% ($P = 0.01$ *vs* PL) CFZ 300 mg = 8.9% ($P < 0.001$ *vs* PL) HOMA-%B = 15.19% to 18.14% (CFZ 100 mg/300 mg, $P < 0.01$ *vs* PL)
		CFZ = 100 mg/300 mg OD + MET + PIO *vs* SITA 100 mg + MET + PIO for next 26 wk	At the end of 52 wk -0.92% to -1.03% (CFZ 100 mg/300 mg)	At the end of 52 wk -26.7 mg/dL to -31.5 mg/dL (CFZ 100 mg/300 mg)	At the end of 52 wk -2.7% to -3.7% (CFZ 100 mg/300 mg)	At the end of 52 wk ΔSBP = -3.4 to -3.7 mmHg (CFZ 100 mg/300 mg) ΔDBP = -2.5 to -2.7 mmHg (CFZ 100 mg/300 mg) ΔHDL = CFZ 100 mg = 7.0% CFZ 300 mg = 11.4% ΔLDL = 10.9% to 14.3% (CFZ 100 mg/300 mg) ΔTG = 4.7% to -0.6% (CFZ 100 mg/300 mg)

Cefalu *et al*[23] CANTATA-SU (*n* = 1450)	T2DM patients with HbA1c: 7%-9.5% on stable MET therapy ⩾ 1500 mg/d, BMI = 22-45kg/m^2, FBG ⩽ 270mg/dL Duration of the study = 52 wk	CFZ = 100 mg/300mg OD + MET *vs* GLIM 6 mg/8 mg OD + MET	-0.82% to -0.93% (CFZ 100 mg/300 mg) GLIM = -0.81%	-1.35 mmol/L to -1.52 mmol/L (CFZ 100 mg/300 mg) GLIM = -1.02 mmol/L	-4.2% to -4.7% (CFZ 100 mg/300 mg, *P* < 0.001 *vs* GLIM) GLIM = 1%	ΔSBP = -3.3 to -4.6 mmHg (CFZ 100 mg/300 mg) GLIM = 0.2 mmHg ΔDBP = -1.8 to -2.5 mmHg (CFZ 100 mg/300 mg) GLIM = -0.1 mmHg ΔTG = CFZ 100 mg = -3.7% CFZ 300 mg = 2.3% GLIM = 9.5% ΔHDL = CFZ 100 mg = 7.9% CFZ 300 mg = 9.0% GLIM = 0.3% ΔLDL = 9.6% to 14.1% (CFZ 100 mg/300 mg) GLIM = 5%
Wilding *et al*[24] CANTATA-MSU (*n* = 469)	26-wk extension study T2DM patients currently treated with MET and SU with HbA1c: 7%-10.5% and FBG < 270 mg/dL	CFZ = 100 mg/300 mg OD + MET + SU *vs* PL + MET + SU	At the end of 26 wk -0.85% to -1.06% (CFZ 100 mg/300 mg, *P* < 0.001 *vs* PL)	At the end of 26 wk -18.2 mg/dL to -30.5 mg/dL (CFZ 100 mg/300 mg, *P* < 0.001 *vs* PL)	At the end of 26 wk -2.1% to -2.6% (CFZ 100 mg/300 mg, *P* < 0.001 *vs* PL)	At the end of 26 wk ΔSBP = CFZ 100 mg = -4.89 mmHg (*P* = 0.07 *vs* PL) CFZ 300 mg = -4.27 mmHg (*P* = 0.2 *vs* PL) ΔTG = CFZ 100 mg = 5.4% (*P* = 0.256 *vs* PL) CFZ 300 mg = 8.5% (*P* = 0.57 *vs* PL) ΔHDL = CFZ 100 mg = 5.7% (*P* = 0.153 *vs* PL) CFZ 300 mg = 6.5% (*P* = 0.056 *vs* PL)
Schernthaner *et al*[25] CANTATA-D2 (*n* = 755)	T2DM patients currently treated with MET and SU with HbA1c: 7%-10.5% and FBG < 300 mg/dL Duration of the study = 52 wk	CFZ = 300 mg OD + MET + SU *vs* SITA = 100 mg OD + MET + SU	CFZ 300 mg = -1.03% (*P* < 0.001 *vs* SITA) SITA = -0.66%	CFZ 300 mg = -29.9 mg/dL (*P* < 0.001 *vs* SITA) SITA = -5.85 mg/dL	CFZ 300 mg = -2.5% (*P* < 0.001 *vs* SITA) SITA = 0.3%	ΔSBP = CFZ 300 mg = -5.06 mmHg (*P* < 0.001 *vs* SITA) SITA = 0.85 mmHg ΔTG = CFZ 300 mg = 9.6% (*P* = 0.554 *vs* SITA) SITA = 11.9% ΔHDL = CFZ 300 mg = 7.6% (*P* < 0.001 *vs* SITA) SITA = 0.6%
Devineni *et al*[26] (*n* = 29)	T2DM patients not optimally controlled on insulin and up to one oral AHA with BMI: 25-45 kg/m^2, FBG: 3.3-5.5 mmol/L, HbA1c: 7%-10.5% and serum creatinine: < 132.6 mmol/L for males and < 123.8 mmol/L for women Duration of the study = 28 d	CFZ 100 mg OD/300 mg bid + Insulin + upto one AHA *vs* PL + insulin + upto one AHA	-0.73% to -0.92% (CFZ 100 mg/300 mg)	-2.11 mmol/L to -2.35 mmol/L (CFZ 100 mg/300 mg)	-0.73 kg to -1.19 kg (CFZ 100 mg/300 mg)	ΔUGE = 71.9 g/d to 129.2 g/d
Rosenstock *et al*[27] (*n* = 451)	T2DM patients under stable MET monotherapy (⩾ 1500 mg/d) with HbA1c: 7%-10%, BMI: 24-45 kg/m^2, serum creatinine: < 1.5mg/dL for males and < 1.4 mg/dL for females Duration of the study = 12 wk	CFZ = 50/100/200/300 mg OD or 300 mg bid + MET *vs* PL + MET *vs* SITA 100 mg OD + MET	CFZ 50 mg = -0.79% 100 mg = -0.76% 200 mg = -0.70% 300 mg = -0.92% 300 mg bid = -0.95% SITA = -0.74%	CFZ 50 mg = -16.2 mg/dL 100 mg = -25.2 mg/dL 200 mg = -27.0 mg/dL 300 mg = -25.2 mg/dL 300 mg bid = -3.4 mg/dL SITA = -12.6 mg/dL	CFZ 50 mg = -2.3% 100 mg = -2.60% 200 mg = -2.70% 300 mg = -3.40% 300 mg bid = -3.4% SITA = -0.60%	ΔSBP = -3.3 to -5 mmHg (CFZ 100 mg/300 mg) SITA = -0.8 mmHg ΔDBP = -1.7 to -2.1 mmHg (CFZ 100 mg/300 mg) SITA = -0.6 mmHg ΔTG = CFZ 100 mg = 2.5% CFZ 300 mg = -2.3% ΔHDL = CFZ 100 mg = 11.2% CFZ 300 mg = 10.6% ΔLDL = 2.9% to 7.1% (CFZ 100 mg/300 mg) HOMA-%B = CFZ 50-300 mg OD = 6% to 18% CFZ 300 mg bid = 16% SITA = 10%

ClinicalTrials.gov identifier: NCT01064414[28] (n = 272)	26-wk extension study T2DM patients with or without AHA, on regular diet and exercise with moderate renal impairment	CFZ = 100 mg/300 mg OD with or without AHA *vs* PL with or without AHA	At the end of 26 wk -0.33% to -0.44% (CFZ 100 mg, P = 0.01 *vs* PL, CFZ 300 mg, P < 0.001 *vs* PL)	At the end of 26 wk CFZ 100 mg = -14.9 mg/dL, P = 0.02 CFZ 300 mg = -11.7 mg/dL, P = 0.06	Information was not available	Information was not available

OD: Once daily; FBG: Fasting blood glucose; PL: Placebo; CFZ: Canagliflozin; ΔSBP: Change in systolic blood pressure from baseline; ΔDBP: Change in diastolic blood pressure from baseline; ΔHDL: Change in blood HDL level from baseline; ΔLDL: Change in blood LDL level from baseline; ΔTG: Change in blood triglycerides level from baseline; ΔPPBG: Change in postprandial blood glucose from baseline; MET: Metformin; SU: Sulphonylurea; SITA: Sitagliptin; PIO: Pioglitazone; ROSI: Rosiglitazone; AHA: Antihyperglycemic agent; IR: Immediate release; GLIM: Glimepride; HOMA-%B: Homeostasis Model Assessment estimating steady state beta cell function in percentage; LDL: Low-density lipoprotein; T2DM: Type 2 diabetes mellitus; PPAR: Peroxisome proliferator activated receptor.

antihyperglycemic agent. Both the CFZ doses (100 mg and 300 mg) showed greater reduction in HbA1c, body weight and FBG[26].

The effects of various doses of CFZ (50, 100, 200, 300 mg OD and 300 mg BD) have also been assessed in a 12-wk trial in T2DM patients under stable metformin therapy. CFZ demonstrated greater reduction in FBG and body weight at all doses as compared to sitagliptin[27].

Three studies conducted trials in special patient population. One study was on adults with T2DM aged 55 to 80 years, not controlled on diet and exercise together with an antihyperglycemic agent. This trial showed that CFZ is equally effective in this age group[15]. In the second study, CFZ showed significant reduction in HbA1c in T2DM patients with or without an antihyperglycemic agent, on regular diet and exercise with moderate renal impairment[29]. The third trial done on T2DM patients with stage 3 chronic kidney disease (CKD) established the safety and efficacy of CFZ in these patients as well[28].

SAFETY PROFILE

Overall, CFZ is well tolerated. The distinctive concern is about increased risk of genital mycotic infections and urinary tract infections (UTI). The data from the four pooled 26-wk placebo-controlled trials including monotherapy trial and three add-on combination trials with metformin, metformin and sulfonylurea or metformin and pioglitazone; demonstrated female genital mycotic infections in 3.2% of patients in placebo, 10.4% in CFZ 100 mg and 11.4% of patients in CFZ 300 mg groups. The incidence of genital mycotic infection was less in males with rates of 0.6% in placebo, 4.2% in CFZ 100 mg and 3.7% in CFZ 300 mg groups. UTI presented at the rate of 4% in the placebo, 5.9% in 100 mg dose and 4.3% in the 300 mg dose groups[15].

Other common adverse events reported were increased urination, vulvovaginal pruritus, thirst, constipation and nausea. The risk of hypoglycemia in patients with CFZ was generally low. Volume depletion-related adverse reactions such as dizziness, hypotension and dehydration were higher in elderly patients, 65 years or older, particularly with the 300 mg daily dose, in comparison to younger patients. There were mild and transient changes in eGFR, albumin-creatinine ratio and blood urea nitrogen in early phase of the study in stage 3 CKD patients. However, 26-wk treatment caused return of these parameters to baseline; there was also an increase in serum potassium and magnesium in these patients[15,29]. Some studies showed increase in LDL-C and hematocrit[15]. These short-term studies have shown no significant changes in vital signs or electrocardiogram finding with CFZ use. A long term prospective study to evaluate the efficacy and adverse effect profile of CFZ is already underway[30]. The summary of adverse events of CFZ is depicted in Table 2.

CONCLUSION

Clinical trial data for CFZ reveal that its glucose lowering efficacy is superior to usual gold standard drugs with the added benefit of weight loss. FDA has already approved CFZ monotherapy in adjuvant to diet and exercise. So far, none of the serious concerns which surround dapagliflozin are seen in CFZ trials. Furthermore, its insulin independent action is an important advantage as this essentially means that its glucose-lowering efficacy should not decrease significantly with progression of diabetes. CFZ is also compatible with other anti-diabetic therapies, including insulin, and might therefore be of value at any stage in the natural history of T2DM. CFZ with its multi dimensional properties can be beneficial in the disease cluster of obesity, hypertension and diabetes. Further, there is a low propensity to cause hypoglycemia in patients as glucose is reab-

Table 2 Summary of adverse events observed in the canagliflozin clinical trials

S. No	ClinicalTrials.gov identifier	Adverse events	Ref.
1	NCT01081834	Increased incidence of AEs in CFZ groups Serious AEs and AE related discontinuations similar in all groups Increased incidence of UTI, genital mycotic infections and osmotic diuresis related AEs in CFZ groups Moderate increase in BUN, serum creatinine and decrease in serum uric acid	Stenlöf *et al*[19]
2	NCT01106677	AEs similar across all groups Higher incidence of pollakiuria in CFZ groups -5.71% with 100 mg CFZ and 2.72% with 300 mg CFZ *vs* 0.55% of PL	[20]
3	NCT01106690	Vulvovaginal mycotic infections: 2.65% to 5.26% *vs* 0% of placebo Pollakiuria: 6.14% to 9.42% *vs* 0.87% of placebo Increased rate of hypoglycemic event with CFZ 300 mg (5.26% *vs* 1.74% of PL)	Guthrie *et al*[21]
4	NCT00968812	Osmotic diuresis related AEs in 3% of CFZ groups as compared to < 1% in placebo groups Genital infections and increase in LDL cholesterol more in CFZ groups	Cefalu *et al*[23]
5	NCT01106625	Superficial genital mycotic infection: 16.0% to 21.0% *vs* 5% in women and 3.4% to 6.6% *vs* 1.3% in men More subjects treated with CFZ had ⩾ 1 hypoglycemic episodes	Wilding *et al*[24]
6	NCT01137812	Genital mycotic infections: 9.2% of CFZ 300 mg *vs* 0.5% of SITA Osmotic diuresis related AEs: 2.4% of CFZ 300 mg *vs* 1.3% of SITA Higher incidence of increased TG in CFZ groups	Schernthaner *et al*[25]
7	Not available	Similar rate of AEs and discontinuations across all groups No serious AEs	Devineni *et al*[26]
8	NCT00642278	Non dose dependent increase in incidence of genital infections (3%-8% *vs* 2% of SITA) and UTI (3%-9% *vs* 2% of SITA) in CFZ groups Low incidence of hypoglycemia Small increase in LDL cholesterol in CFZ groups	Rosenstock *et al*[27]
9	NCT01064414	AEs similar across all groups Increased incidence of hypoglycemic events in CFZ groups -14.44% with 100 mg CFZ and 11.24% with 300 mg CFZ *vs* 4.44% of PL	[28]

AEs: Adverse events; CFZ: Canagliflozin; UTI: Urinary tract infections; SITA: Sitagliptin; LDL: Low-density lipoprotein; PL: Placebo; TG: Triglycerides; BUN: Blood urea nitrogen.

sorbed by SGLT1 in kidney. In addition to the reported side effects of CFZ like UTI, genital mycotic infections, volume depletion and hypotension, the high cost of CFZ may prove to be a limiting factor in its wide spread use. However, for the time being CFZ has been proven to be safe and well tolerated and it is for the further long term studies to establish it more firmly as a major breakthrough in the clinical armamentarium for patients with diabetes.

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P- Reviewers: Georgescu A, Tamemoto H **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Structured SMBG in early management of T2DM: Contributions from the St Carlos study

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Received: January 13, 2014 Revised: April 11, 2014
Accepted: May 29, 2014
Published online: August 15, 2014

Abstract

Diabetes mellitus type 2 (T2DM) is a global pandemic that will affect 300 million people in the next decade. It has been shown that early and aggressive treatment of T2DM from the onset decreases complications, and the patient's active role is necessary to achieve better glycemic control. In order to achieve glycemic control targets, an active attitude in patients is needed, and self-monitoring of blood glucose (SMBG) plays a significant role. Nowadays, SMBG has become an important component of modern therapy for diabetes mellitus, and is even more useful if it is performed in a structured way. SMBG aids physicians and patients to achieve a specific level of glycemic control and to prevent hypoglycemia. In addition, SMBG empowers patients to achieve nutritional and physical activity goals, and helps physicians to optimize the different hypoglycemic therapies as demonstrated in the St Carlos study. This article describes the different ways of using this educational and therapeutic tool from the medical point of view as well as from the patient's perspective.

Key words: Structured self-monitoring of blood glucose; Educational; Therapeutic; Tool; Management; Diabetes mellitus type 2

Core tip: Structured self-monitoring of blood glucose (SMBG) has recently become an important component of modern therapy for diabetes mellitus due to its educational and therapeutic role. SMBG aids physicians and patients to achieve a specific level of glycemic control and to prevent hypoglycemia. It empowers patients to achieve nutritional and physical activity goals, and helps physicians to optimize the different hypoglycemic therapies as demonstrated in the St Carlos study.

Original sources: Ruiz Gracia T, García de la Torre Lobo N, Durán Rodríguez Hervada A, Calle Pascual AL. Structured SMBG in early management of T2DM: Contributions from the St Carlos study. *World J Diabetes* 2014; 5(4): 471-481 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/471.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.471

INTRODUCTION

Diabetes mellitus is known by a number of syndromes that are a consequence of a lack of insulin secretion or by a defect in its hypoglycemic action. Hyperglycemia is the common feature in all of these syndromes, and if it is present for a long period of time it can cause vascular damage. Despite the significant development in hypoglycemic drug therapies over the past two decades, diabetes remains the leading cause of new cases of blindness, kidney failure, and limb amputations not related to accidents or injury in adults. Moreover, the incidence and prevalence of this disease continues to increase, as a result of an unhealthy and sedentary lifestyle in developed coun-

Table 1 Targets of glycemic control

	IDF	AAEC	ADA	St Carlos study
HbA1c (%)	< 6.5	≤ 6.5	< 7.0	< 6.5%
Fasting/preprandial glycemia (mmol/L-mg/dL)	< 6.0/< 110	< 6.0/70-110	3.9-7.2/70-130	< 6.0/< 110
2-h postprandial glycemia (mmol/L-mg/dL)	< 7.8/< 140	< 7.8/< 140	< 10.0/< 180	< 7.9/< 145

IDF: International Diabetes Federation; AACE: American Association of Clinical Endocrinologists; ADA: American Diabetes Association.

tries, and is nowadays considered a pandemic disease. According to the International Diabetes Federation (IDF), in the next decade it will affect more than 300 million people worldwide. The incidence and severity of complications depend mainly on metabolic control and time to disease progression. Therefore, an early and individualized approach to achieve strict glycemic control is needed along with the management of other cardiovascular risk factors. To achieve this aim, it is essential that patients with diabetes assume an active role in their care, and self-monitoring of blood glucose (SMBG) plays a significant role.

In the early 1990s, the first meter for self-monitoring capillary blood glucose was released. In many researchers' opinion it was the greatest research after that on insulin. SMBG increases life expectancy and improves diabetic patients' quality of life. The Diabetes Control and Complications Trial[1] showed that its use as an educational and therapeutic tool significantly reduced complications and delayed existing complications in type 1 diabetes mellitus (T1DM). To date, the intensive treatment of diabetes consists of multiple daily injections of insulin, however, later this concept was extended to include multiple glucose capillary determinations conducted by the patient in order to perform multiple self-treatment adjustments (including oral drugs and insulin). In T2DM, the results have been more controversial, especially in patients not treated with insulin. However, our group showed that the use of SMBG in an educational program increased the regression rate in newly diagnosed type 2 diabetic patients and led to changes in lifestyle and weight loss[2].

The success of this technique is due to the empowerment that SMBG provides to patients. SMBG shows variations throughout the day facilitating decision-making on changes in hypoglycemic treatment as well as lifestyle at particular time points. These features make SMBG not only a good tool for glycemic control, but also a good tool to prevent hypoglycemia, to improve the quality of life of diabetic patients and for better management of economic resources.

TARGETS OF GLYCEMIC CONTROL

Both patients and health care staff need to jointly agree on the terms and use of SMBG. This can change depending on lifestyle and the pharmacological treatment provided. It is recommended that targets are set by individual steps. The main objective is to achieve normal glycemia values or very close to the normal standards with hemoglobin A1c (HbA1c) levels below 7%. These targets decrease micro-vascular complications as shown in different studies[2,3]. A stricter regime (*i.e.*, level below 6.5%) can be considered for specific patients (as long as it does not result in adverse effects or severe hypoglycemia) with a high life expectancy rate and short disease evolution. A higher glycemic objective (below 8%) may be appropriate for patients with a limited life expectancy, comorbidities and complications, and for those with severe hypoglycemic risk[4]. For this reason, it is necessary to individualize the treatment in line with the patient's "biological" age[5]. We should bear in mind that the HbA1c parameter for glycemic exposure for the last three months might not be as relevant as is currently believed. Other parameters, such glycemic variability, are becoming a significant risk factor involved in the pathogenesis of diabetes complications[6,7]. For example, patients with similar levels of HbA1c can show variability in cardiovascular risk, which indicates that there are unknown factors involved. For this reason, it should be common practice to carefully consider SBMG, as it shows real-time variability of blood glucose.

With regard to glycemic objectives, the ADA and EASD recommendations for glycemic targets[4,8] are shown in Table 1.

Our working group has assumed the same targets as those in the St Carlos study[9]. When objectives in at least 60% of the registered capillary blood tests are not achieved, it is time to take action, either drug titration or introducing new drugs (this theme is further developed in the following section: glycemic assessment and then taking action).

SELF-MONITORING BLOOD GLUCOSE: WHAT IS IT?

This self-analysis is defined as the self-measurement of capillary blood glucose by the patient using an accurate device, digital or battery-operated, that measures capillary glucose in real time. The aim of SMBG is to collect detailed information on glucose levels at many time points during the day in order to implement various strategies to fit the patient's lifestyle. It can be used to guide a new regimen, and it can help people day-to-day to adjust their food intake, physical activity, and their dose of insulin to improve glycemic control.

This useful tool represents the highest level of patient participation. The best decision-making occurs when patients reach a higher level of knowledge and skills to

adhere to changes in lifestyle; similarly, they make proper use of hypoglycemic drugs. Thus, SMBG should be established from the onset to guide initial treatment to ensure better glycemic control.

SMBG could complement HbA1c testing, however, the following factors should be considered: it distinguishes between fasting, before meals, and postprandial hyperglycemia. Glycemic excursions are detected early. It identifies hypoglycemia and its resolution by providing immediate feedback on food choices, activity and different medications.

Methodology of SMBG

The test involves pricking a finger with a lancet device to obtain a tiny blood sample and apply this on a test strip. Subsequently, the blood glucose concentration is determined by inserting the strip into a reflectance photometer for automatic reading. Thus, subjects with diabetes are taught to learn from the results and make corrections by changing their intake of carbohydrates, by changing their physical activity or by changing the dose of medication.

Advantages of SMBG

To perform SMBG the patient does not require help and it can be carried out anywhere. SMBG provides immediate accurate data, which can help patients and their relatives in the daily management of diabetes and can teach them to face new future events. The other important advantages of SMBG should be highlighted. SMBG informs patients whether their treatment is working and guides the health care team on whether to continue with the same treatment regimen or if another treatment is needed. The structured SMBG strategy may help patients in their daily routine to maintain a blood glucose level as normal as possible with proper food choices (with a low or high amount of carbohydrates) and with proper life-style choices. It should also be pointed out, that SMBG improves recognition of either severe hyperglycemia or hypoglycemia. This increases the understanding of hypoglycemia and helps reduce anxiety regarding hypoglycemia. Moreover, SMBG is important for the performance of hazardous tasks which could be influenced by high or low glycemic levels, such as driving or operating machinery.

Disadvantages of SMBG

The disadvantages of SMBG are mainly related to the patient who may have a lack of motivation for testing or does not have enough education on how to interpret his own results or does not know when they should be performed. In this case, the following disadvantages may outweigh the potential benefits. SMBG may increase anxiety regarding glycemic control which is closely related to state of health. Other negative aspects to bear in mind are as follows: the pain derived from finger prick and the cost of testing supplies, whether they have to be self-funded or not.

Obviously, a single system of SMBG does not meet the needs of all people with T2DM, thus it must be adapted according to different patients' characteristics. For instance, meters in elderly patients should be simple and manageable and in blind patients they should incorporate sound alarm systems.

FREQUENCY OF SMBG

The frequency of SMBG is a critical point in treatment efficiency, therefore, SMBG protocols should be individualized according to patient characteristics, needs and changes in lifestyle and treatments. The frequency of SMBG also depends on the availability and expertise of the health care team. The program should intensify in frequency in cases of suboptimal glycemic control and changes in lifestyle or treatment. When possible, the fewest determinations should be carried out to allow appropriate adjustment of treatment. In addition, it is important to emphasize that not only patients should collect and interpret the results, but the health care team should also interpret the glucose readings and act accordingly.

As mentioned previously, glycemic targets must be agreed by the patient and their physician. Ideally, patients should achieve goals of glycemic control as close as possible to the value of those without diabetes. Determinations should be performed before each meal and 2 h after eating, and whenever there is risk of hypoglycemia, especially at night (which is the time with the highest risk of hypoglycemia). Therefore, a complete profile will include the identification of at least 6 points if three meals a day are consumed.

Based on the St Carlos study[9], in patients with newly diagnosed T2DM, the following strategy was proposed: The profile consisted of six points if three main meals were consumed daily. The frequency may vary depending on the stability of the patient, as shown in Table 2. It is noteworthy that the strategy proposed by our group has also been adopted in several European consensus documents[10,11] which have subsequently been published. Therefore, the role of the structured SMBG in the management of diabetes has been confirmed.

At the onset of disease, the frequency of this strategy (six point profile) should be twice a week and evaluated every five complete profiles to adopt changes in treatment. This frequency must be maintained to achieve stability. Stability is achieved when no changes in three consecutive visits are observed, thus, the frequency can be reduced to one profile once every two weeks in order to maintain adherence to the treatment plan. When there is a risk of suboptimal glycemic control, intercurrent diseases or changes in lifestyle, the frequency should increase and self-testing should be performed as many times as necessary. However, if the patient is treated with continuous subcutaneous insulin infusion he will require at least a four point profile daily, although a seven point profile is recommended, based on the frequency of food intake.

It is important to inform patients that these profiles, if they are not carried out during their everyday lifestyle,

Table 2 Frequency of self-monitoring of blood glucose

	Breakfast		Lunch		Dinner		Night	Periodicity
	Before	After 2 h	Before	After 2 h	Before	After 2 h		
At the onset of T2DM	a	a	a	a	a	a		2-3 d/wk
Suboptimal control of T2DM	a	a	a	a	a	a		2-3 d/wk
T2DM targets in	a	a	a		a			1 d every 7-14 d
Insulin-treated T2DM in the adjustment phase	a	a	a	a	a	a	Each 3 risk profiles	Daily
Insulin-treated T2DM in the education programs	a	a	a	a	a	a	Each 3 risk profiles	Daily
Insulin-treated	a	a	a	a	a	a	Each 3 risk profiles	2-3 d/wk
T2DM targets in GDM	a	a	a	a	a	a		Daily

[a]Specific time of day in which self-monitoring of blood glucose should be performed. T2DM: Diabetes mellitus type 2; GDM: Gestational diabetes mellitus.

may not be as useful as they could be for the health team to make decisions on therapy. Thus, we do not recommend SMBG during medical consultation, as this is probably not a usual day in the patient's life. Recently, a structured program was proposed, which consists of three consecutive profiles prior to the medical visit to make decisions on treatment[12]. This strategy has proven to reduce absolute values of Hb1AC by 1.2%.

SMBG IS A THERAPEUTIC TOOL TO IMPROVE GLYCEMIC CONTROL

Although the benefits of SMBG have been demonstrated in T1DM[1] and insulin-treated T2DM[13-15], findings from SMBG studies in non-insulin-treated T2DM[16-20] have been inconsistent. As a result of this, the IDF has recently published a guide for SMBG in non-insulin treated subjects with diabetes[21]. In this guide, the IDF recommends that SMBG should be implemented only when patients and/or their physicians have the knowledge, skills and willingness to incorporate self-analysis into their routines in order to achieve the agreed objectives of treatment. This emphasizes the need for collaboration between the patient and the treating medical team to act jointly.

The study conducted by Evans *et al*[22] demonstrated a statistically significant correlation between the number of daily SMBG tests performed and HbA1c levels. It was observed that patients who performed SMBG more than once per day showed a reduction in HbA1c of 0.7%. Furthermore, to reduce HbA1c levels below 7% it was necessary to carry out SMBG at least six times a day[22]. The results of the St Carlos study were similar. Newly diagnosed T2DM patients were randomized to either a structured SMBG-based intervention (n = 130) or an HbA1c-based control group (n = 65) and were followed for 3 years. The primary endpoint was the regression rate of T2DM. Diabetes regression was observed to be 4.5 times more likely in the intervention group, and that this was associated with greater adherence to dietary and physical activity recommendations. Moreover, a greater weight loss of 4 kg was 3.6 times more likely in the intervention group. The study included a three-year follow-up period, and indicated that the benefits of a structured SMBG program are maintained long-term[2]. Results from the ROSSO[23] and the PRISMA studies[24] support our results.

Therefore, SMBG is not a treatment, but a tool which provides data to adjust treatment. Changes in therapy can be made as soon as the values for SMBG are obtained and before they have an effect on HbA1c. Consequently this useful and efficient tool must be accessible in both primary care and diabetes care centers.

SMBG AS AN EDUCATIONAL TOOL

The active participation of subjects with diabetes in the control and treatment of their disease is an essential component of diabetes care. To that purpose, it is necessary that those with diabetes have an adequate level of knowledge and skills to make proper decisions on their treatment. Through an educational program, diabetics can gain the necessary knowledge, skills and motivation to modify, adopt and maintain healthy behaviors and positive attitudes toward self-management.

Within this context, SMBG is a very handy tool which helps patients understand the disease. In particular, SMBG shows variations in blood glucose in a single day, for instance during exercise, meals, physical and emotional stress. This tool encourages self-management of diabetes[25], allowing patients to measure the impact of their behavior (the effect of eating reflected in postprandial glucose, *etc*.) thus promoting greater adherence to dietary and exercise advice in their daily lives.

In addition to its educational role, SMBG is a powerful motivating factor. It provides positive feedback on the success or failure after making self-adjustments. This can lead to increased confidence in patients to be more self-sufficient, more responsible and can make them more involved in the disease.

However, the DiGEM study[26], did not observe benefits from SMBG in patients with non-insulin-treated T2DM. There are several noteworthy aspects in this study which were crucial in obtaining these data. All treatment changes were performed by physicians, regardless of the

Table 3 Nutrition and activity score

	Score		
	+1	0	-1
Physical activity			
Walking daily (> 5 d/wk)	> 1 h	At least 30 min	< 30 min
Climbing stairs (No. floors/d, > 5 d/wk)	> 16	4-16	< 4
At least 30 min of more than moderate intensity	> 3 d/wk	2 or 3 d/wk	< 2 d/wk
Servings per week			
Vegetables	> 12	6-12	< 6
Fruits (pieces)	> 12	6-12	< 6
Nuts	> 3	1-3	< 1
Olive oil	Daily	> 3 d	< 3 d
High-fat fish or Iberico ham	> 3	1-3	< 1
Bread and cereals (high fiber content)	> 6	3-6	< 3
Legumes	> 2	1-2	< 1
Low-fat milk and cheeses	> 6	3-6	< 3
Red meat	< 3	3-6	> 6
Sauces (except mayonnaise)	< 2	2-4	> 4
Juices and sugar-sweetened beverages	< 2	2-4	> 4
Cookies	< 2	2-4	> 4
Coffee	> 3/d	< 3	> 4
Alcoholic beverages (No. servings/d)	1-4	0 or > 4 and < 6	> 6
Water	Exclusively	In addition to other beverages	Never

team of nurse educators. In addition, the patients had experienced more than 3 years of diabetes progression when they entered the study, so they were less receptive to this educational tool due to apathy. Thus, we believe that this tool is very helpful from disease onset to provide a greater educational effect, and it is at this point that it is crucial to apply an integrated program based on SMBG. This may explain the conflicting results with our study.

GLYCEMIC ASSESSMENT AND THEN TAKING ACTION

Currently, only invasive procedures, such as subcutaneous continuous glucose monitoring and SMBG, can provide accurate information on the daily profile of blood glucose levels.

The magnitude of the variation in glucose has proved to be the most reliable factor associated with the increased risk of severe hypoglycemia[27] and has been associated with subsequent microvascular and macrovascular complications[28-31]. Hence, the concept of glycemic variability is very important as it is one of the major features of T2DM. SMBG is recorded in real time, but HbA1c is not. Thus, this tool provides information for both patients and doctors, and on lifestyle changes if needed, in order to achieve better glycemic control. Furthermore, it also allows the physician to make adjustments to the different doses of oral hypoglycemic drugs or insulin, depending on the levels registered, to avoid hypoglycemia and hyperglycemia.

To take action, we should take into account that each determination of capillary glucose is explained by previous events. Each determination assesses previous events, such as, the effect of food ingested previously, exercise performed earlier and the dose of drug administered previously. Glycemic variability is explained in more than 90% of cases by food intake. For this reason and in order to achieve targets, it would be advisable to wait at least 3 out of 5 profiles performed in similar conditions to make changes to the diet, or to make changes in hypoglycemic drugs if needed. Therefore, therapeutic changes are required if more than 60% of blood glucose levels are off target, both above and below. In addition, the patient should determine possible reasons for these values. It is recommended that these interpretations should be transcribed into the book of patients' profiles and later discussed during the medical visit with the health care team, both the physician and diabetes educator. Therefore, we stress the importance of correct collection of self-analysis, as data which are not transcribed cannot be evaluated in order to make changes.

Glycemic assessment conducted by the medical team: A proposal of changes in lifestyle and changes in therapy and dose of hypoglycemic drugs

After establishing the diagnosis of T2DM, the physician and the patient must agree therapeutic targets as well as changes in the patient's lifestyle. After 3-6 mo of non-response, pharmacological treatment should be initiated[4,8]. To achieve success, patients must be informed regarding a healthy lifestyle (Table 3).

Interventions in lifestyle include: smoking cessation, dietary and exercise prescription and diabetes education to change negative attitudes and promote healthy lifestyles. All these recommendations are in order to reduce cardiovascular morbidity and mortality in patients with T2DM.

Before adjusting treatment the following factors should be determined: (1) If in three out of five profiles the fasting blood glucose or the postprandial blood glucose values remain within target the patient should remain on the same treatment recommendations; (2) If the target levels are above the objective levels in 60% of cases (3 of 5) the following are recommended: lifestyle recommendations should be intensified. The patient should assess his intake (focused on carbohydrates) and if possible try to decrease the amount of carbohydrates in order to control postprandial glycemia. Another option might be to recommend an increase in physical activity before meals as exercise increases insulin sensitivity; with regard to hypoglycemic drugs, these should be titrated or a new drug added. We first add insulin sensibilizator Should this be sensitizing drugs (metformin or pioglitazone) at the maximum tolerated doses. If the targets are not reached we add drugs based on secretory insulin action (sulfonylurea, glinides, gliptins, glucagon-like peptide-1 agonists or insulin); and (3) If glucose levels are below 70 mg/dL, there are two options: ask the patient to adjust carbohydrate

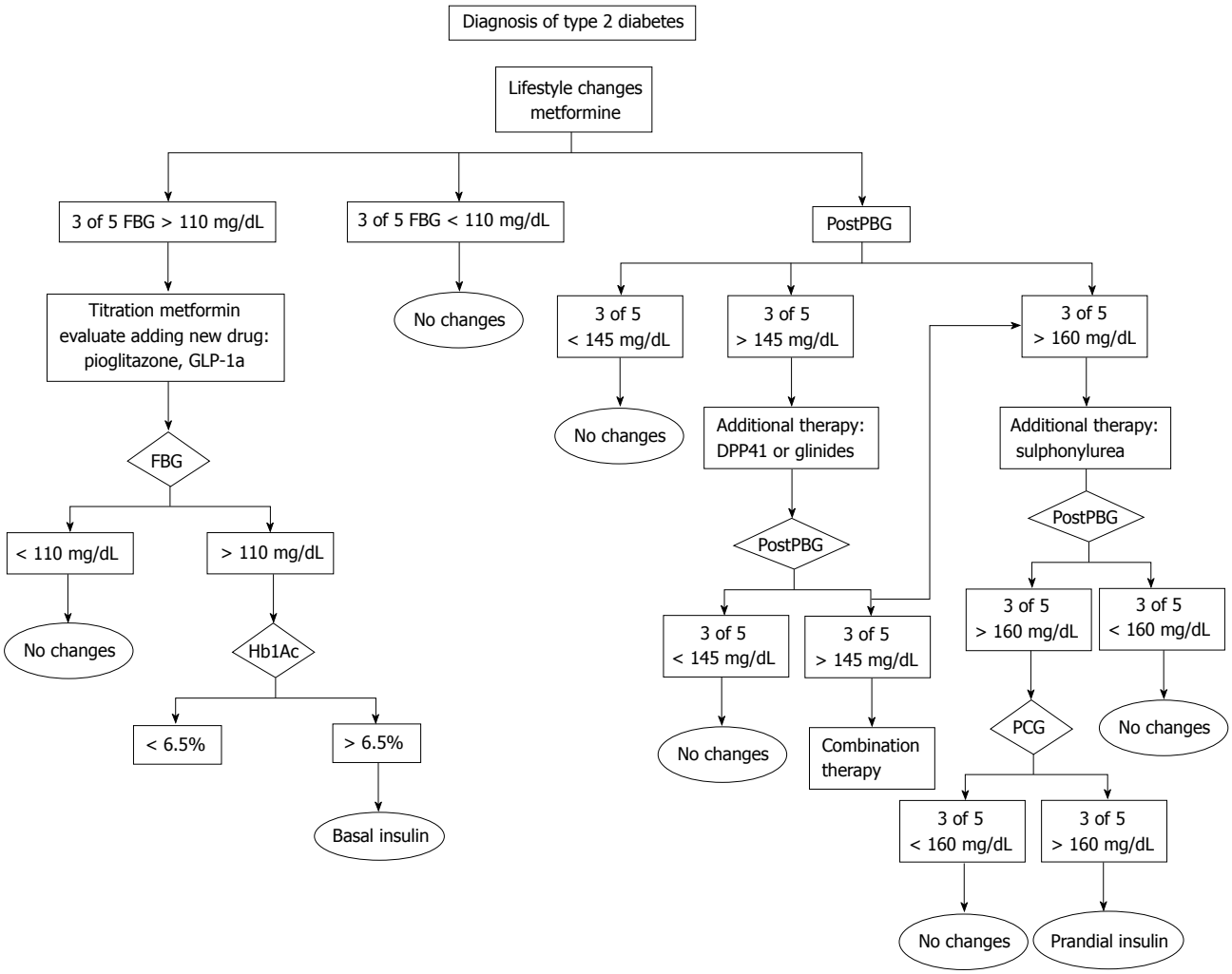

Figure 1 Decision algorithms based on self-monitoring of blood glucose from the diagnosis of type 2 diabetes mellitus as proposed in the St Carlos study. FBG: Fasting blood glucose; GLP-1a: Glucagon-like peptide-1 agonists; PostPBG: Postprandial blood glucose; FBG: Fasting blood glucose; PCG: Titration sulphonylurea.

intake or reduce the dose or the number of drugs prescribed. Figures 1-4 show different algorithms for adjusting diabetes treatment.

Glycemic assessment conducted by patients: Changes in lifestyle and adjustment of doses of hypoglycemic drugs

Due to the educational role of SMBG, patients can be self-sufficient, adequately responding to glycemic fluctuations under different situations, and achieving results very close to the agreed targets.

Fasting glucose assessment: Fasting glucose is the existing glycemia prior to breakfast or eight hours after fasting. This type of glycemia shows minimal pharmacological and intake interference, and shows the effect of gluconeogenesis.

The main causes of fasting hyperglycemia are related to the following: (1) medical prescription errors: the prescribed medication dosage is too low, timing of administration may be inappropriate, or the medication does not effectively target fasting pre-prandial glycemia. Our recommendation is to increase the dose of drugs if hyperglycemia persists for three consecutive days in the daily profile. For instance, if basal insulin is administered during the afternoon or in the evening, patients should increase their usual dose of basal insulin as recommended by their physician without waiting for medical consultation. To do so, patients must be adequately trained; and (2) patient behavior: incorrect medication administration (dosage errors, inappropriate timing), failure to take medication, *etc*. Frequently, we observe a wrong tendency in patients of making changes based only on the registered glycemia (high or low). This is known as rescue therapy. This attitude would be valid only to correct an unforeseen specific situation and to avoid the consequences of sustained hyperglycemia or hypoglycemia. However, this attitude should not be allowed to continue, and an analysis of previous events should be carried out to make appropriate changes if needed. To improve a patient's skills it is essential to have a good team of diabetes educators in order to improve knowledge and glycemic control.

Pre-prandial glucose assessment: Pre-prandial glycemia evaluates previous food intake, which means:

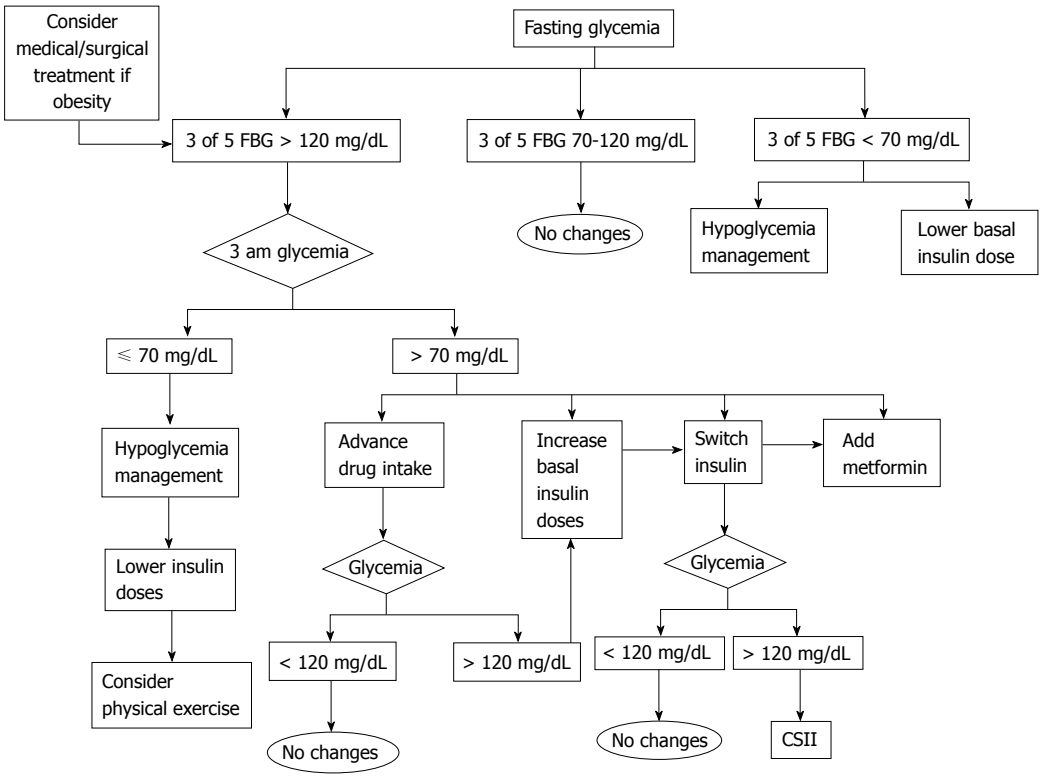

Figure 2 Decision algorithms based on fasting self-monitoring of blood glucose in the evolution of type 2 diabetes mellitus as proposed in the St Carlos study. FBG: Fasting blood glucose; CSII: Continuous subcutaneous insulin infusion.

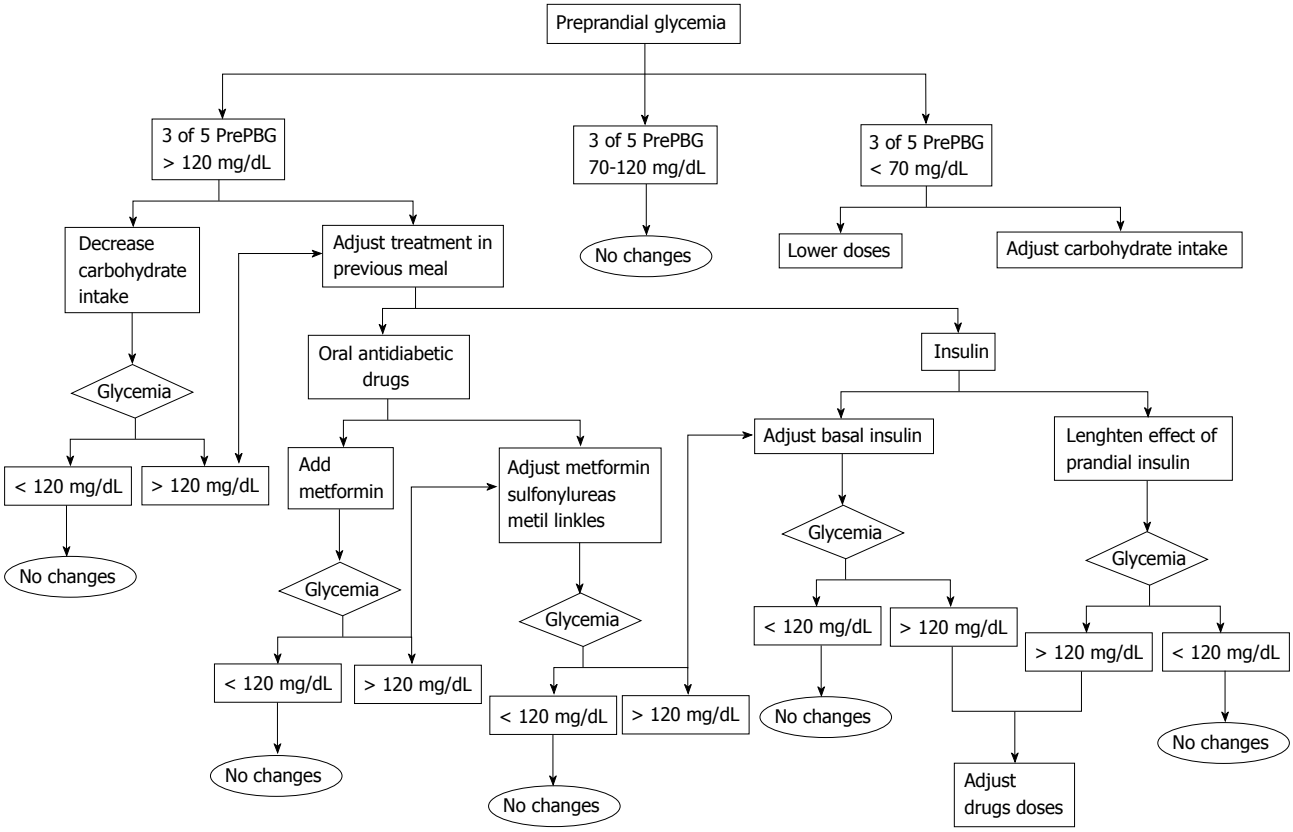

Figure 3 Decision algorithms based on preprandial self-monitoring of blood glucose in the evolution of type 2 diabetes mellitus as proposed in the St Carlos study. PrePBG: Preprandial blood glucose.

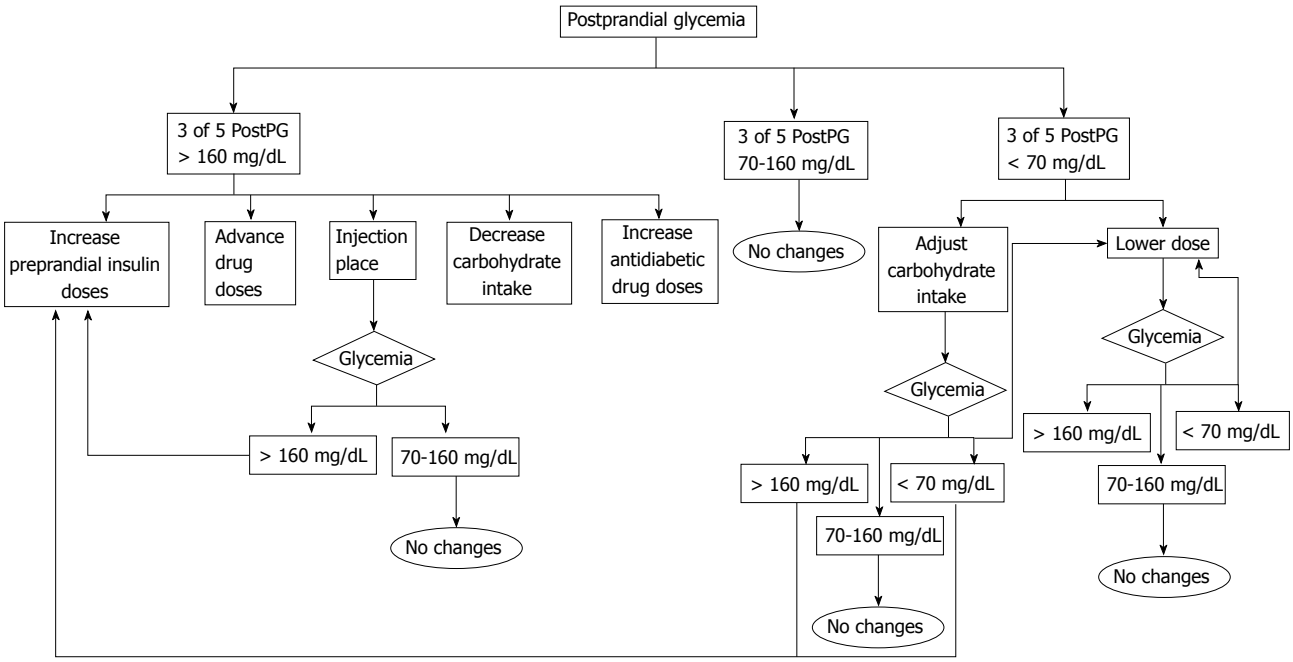

Figure 4 Decision algorithms based on postprandial self-monitoring of blood glucose in the evolution of Type 2 diabetes mellitus as proposed in the St Carlos study. PostPG: Postprandial blood glucose.

mid-morning or afternoon snack, as well as any physical activity conducted before the analysis. A nutritional recommendation might be to decrease the intake of meat (sausage, bologna, ham, salami, *etc.*), cheese, all types of manufactured products, French fries, *etc.* Our recommendation is to substitute those snacks for a limited intake of nuts. Nuts such as almonds, walnuts and hazelnuts have a lower glycemic index and substantially reduce unhealthy fats and they provide mono and polyunsaturated fats (fatty acids oleic, linoleic and omega 3 fatty acids) with high benefits shown in previous reviews[32]. In addition, nuts satiate the appetite and improve microbiota. In addition, promoting physical activity at this point will improve insulin sensitivity.

Postprandial glucose assessment: We evaluate glucose two hours after breakfast, lunch and dinner: (1) in general terms, if glycemia is above the target, we propose one of the following options: reduce the amount of carbohydrate intake, substitute common foods for lower glycemic foods (*i.e.*, white bread for wholewheat bread), modify antidiabetic treatment (*i.e.*, increase prandial insulin) and perform physical activity after food intake; and (2) in those cases with hypoglycemia (< 70 mg/dL), patients are recommended to put into practice the protocol advised in order to resolve hypoglycemia. They will also have to analyze what triggered that specific glycemic level (*i.e.*, insufficient intake of carbohydrates, too much exercise or inadequate drug doses).

The following three questions may be useful in analyzing postprandial glycemia and in understanding the root of the problem in order to act accordingly: (1) what did the patient eat? The patient must analyze what he ate two hours previously, identify foods with high glycemic index and avoid them or substitute them for other foods with a low glycemic index in the coming days; (2) when did the patient eat it and when was the self-analysis performed? The patient should record when he carried out the self-analysis so that the results regarding glucose intake can be put into context. If capillary glucose levels are low after two hours or more, two options are available: increase the intake of slow-absorption carbohydrates or bring forward the next meal; and (3) how did the patient eat it? We know that the way food is cooked is the key to its absorption, for this reason it is important that the patient is informed regarding this. For instance, for the same amount of potatoes, fried potatoes significantly increase the glycemic index, whereas, boiled potatoes show a lower postprandial increase.

Postprandial glucose assessment after breakfast: Postprandial glycemia after breakfast provides information on the foods which are rich in carbohydrates. In cases where glycemia is high we can choose any of the options mentioned above. Recently it was shown that juices, even natural juices, have a high glycemic load, so they are not as healthy as expected. For this reason, we, as professionals, need to educate the diabetic population, that juice intake is inappropriate. Breakfast might also be a good time to evaluate the response to biscuits, including wholewheat biscuits, many of which contain saturated fats. To ensure a healthy breakfast we recommend substituting juice for a piece of fruit, wholewheat bread instead of white bread, and the addition of olive oil to bread instead of ham or butter.

Postprandial glucose assessment after meals: Postprandial glycemia after meals provides information on the

foods rich in carbohydrates and the way food has been cooked. Similar to breakfast time, in those cases where glycemia is high, we can choose any of the options mentioned above to decrease the level of glycemia (Ⓐ). High levels of glycemia are mostly associated with cereal intake, basically white bread and white rice and food containing potatoes (*i.e.*, French fries, Spanish omelet). For this reason, it is advisable to introduce salads and vegetables as starters, and a piece of fruit for dessert. These are recommended daily foods with limited glycemic load and they also lead to satiation. These foods are also recommended when body weight has become a significant issue.

Glycemic assessment during illness

During the presence of disease it is required that patients increase self-analysis, and adjust the treatment according to the results. For instance, during vomiting patients must consume sugar-containing fluids (juices, milk, isotonic drinks *etc.*) to avoid hypoglycemia. If this is not controlled, patients should look for assistance.

QUALITY OF LIFE AND SMBG

The St Carlos study[9] also assessed treatment satisfaction regarding interference with quality of life (family, social and labor). Initially, patients in the intervention group showed greater interference and stated that it was an added challenge to correctly perform SMBG. However, after a year of follow-up, they reported a greater degree of independence in the three different areas (family, social and labor) and a greater degree of satisfaction with the treatment plan compared to the control group. These data persisted after three years of follow-up.

The explanation for this appears to be simple. When SMBG is integrated into the treatment plan, it can tailor treatment to the patient's lifestyle. In addition, patients who do not know about this tool have to change their lifestyle in order to adapt it to the treatment plan, significantly reducing their index of satisfaction.

Not all patients attain self-sufficiency, including most elderly people with social, family or cultural constraints, and some T2DM patients on conventional treatment. Other studies suggest that this tool produces increased stress in the patient associated with the determination of glycemia and frustration over poor results, especially if the patient does not know how to respond.

Therefore, SMBG when integrated into a comprehensive educational program most likely improves the quality of life of patients by allowing them to self-sufficiently manage their daily lives.

COST IMPLICATIONS OF SMBG, PROS AND CONS

Due to the relatively high cost of SMBG, particularly the use of test strips, it would be remiss to ignore the economic implications. Therefore, it is necessary to balance the benefits of SMBG against its costs.

The implementation of this tool from the onset of disease has benefits for glycemic control that will lead to a decrease in chronic diabetes complications. SMBG is costly in the short-term, but may not be so costly in the long-term, as it helps to reduce the treatment costs of the chronic complications of diabetes through improved glycemic control. Accordingly to a recently published Spanish study[33] conducted in the autonomous community of Madrid, the average cost of T2DM complications per patient was estimated to be 4121.54 Euros (66% due to macrovascular complications), whereas the cost of the test strips only accounted for 2% of the expenditure. Thus, SMBG it is an efficient tool in the treatment of diabetes.

CONCLUSION

SMBG is an essential tool in insulin-treated T2DM, and as shown in this article, in non-insulin treated T2DM. SMBG should be an integral part of the treatment in newly diagnosed T2DM patients. It enables patients to adapt their lifestyle more effectively to achieve better glycemic control and provides insights into patients and clinicians concerning the effectiveness of therapies in glycemic control. Despite this, none of the current guidelines include SMBG in their algorithms, and it is necessary to change this point of view. We advocate the implementation of structured-SMBG in newly diagnosed T2DM, as SMBG is a key element for decision-making in hypoglycemic therapy (lifestyle changes and drugs).

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P- Reviewer: Kumar KVS **S- Editor**: Ji FF
L- Editor: Webster JR **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Effects of exercise training on mitochondrial function in patients with type 2 diabetes

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Received: December 16, 2013 Revised: May 23, 2014
Accepted: June 10, 2014
Published online: August 15, 2014

Abstract

Type 2 diabetes is characterized by a decreased ability of insulin to facilitate glucose uptake into insulin sensitive tissue, *i.e.*, skeletal muscle. The mechanism behind this is at the moment unresolved. It has been suggested that increased amount of lipids inside the skeletal muscle (intramuscular triglyceride, diacylglycerol and ceramides) will impair insulin action in skeletal muscle, but data are not consistent in the human literature. It has also been hypothesized that the impaired insulin sensitivity is due to a dysfunction in the mitochondria resulting in an impaired ability to oxidize lipids, but the majority of the literature is not supporting this hypothesis. Recently it has been suggested that the production of reactive oxygen species play an essential role in skeletal muscle insulin sensitivity. It is well accepted that physical activity (endurance, strength and high intensity training) improves insulin sensitivity in healthy humans and in patients with type 2 diabetes. Whether patients with type 2 diabetes have the same beneficial effects (same improvement) as control subjects, when it comes to regular physical activity in regard to mitochondrial function, is not established in the literature. This review will focus only on the effect of physical activity on skeletal muscle (mitochondrial function) in patients with type 2 diabetes.

Key words: Mitochondria; Exercise; Type 2 diabetes

Core tip: It is well described that exercise interventions improves insulin sensitivity and maximal oxygen uptake in patients with type 2 diabetes as well as in control subjects. When it comes to adaptations in mitochondrial function after an exercise intervention the literature is more sparse especially in patients with type 2 diabetes. Furthermore the medication that patients with type 2 diabetes are using, are often not described well in the papers, and it is known that the different medication (statins and antihypertensive agents) have a major effect on mitochondrial function and insulin sensitivity.

Original sources: Larsen S, Skaaby S, Helge JW, Dela F. Effects of exercise training on mitochondrial function in patients with type 2 diabetes. *World J Diabetes* 2014; 5(4): 482-492 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/482.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.482

INTRODUCTION

The pathophysiology of type 2 diabetes involves the secretion and the action of insulin. The prevailing view[1] is that an inability of insulin to exert its action on the central target tissues, skeletal muscle (mediate glucose uptake initialized by the binding of insulin to its receptor), adipose tissue (mediate glucose uptake and inhibit lipolysis) and hepatic tissue (inhibit glucose output), results in increasing concentrations of glucose in the blood. In response, insulin secretion from the pancreatic beta-cells

is increased, and hyperinsulinemia prevails. Only in those patients in whom the enforced production of insulin from the pancreas fails, hyperglycemia develops and overt type 2 diabetes becomes apparent. The mechanism for the failing pancreatic insulin production is not resolved, while the development of impaired insulin action (insulin resistance) is linked to the development of obesity (in particular visceral fat) and a physical inactive lifestyle and the molecular mechanism is being unraveled these years[2]. Type 2 diabetes is also frequently seen in a cluster of pathologies, including hypertension, endothelial dysfunction, and obesity. Complications to type 2 diabetes include macrovascular complications (atherosclerosis), but also microvascular complications such as neuropathy, nephropathy, retinopathy and angiopathy are known to occur in these patients.

In the past decade mitochondrial dysfunction in skeletal muscle has been linked to insulin resistance[3-7], but an agreement has not been reached and the majority of data does not support this notion[8-17]. It has been shown that patients with type 2 diabetes have 30% lower mitochondrial content in their skeletal muscle compared to healthy control subjects[12,18], and yet the intrinsic mitochondrial function (*i.e.*, respiratory rates normalized for mitochondrial content) is similar in these two groups[12-14]. As such the suggested scenario with insulin resistance being induced by mitochondrial dysfunction *via* accumulation of lipids and lipid intermediates, interfering with insulin signaling[19], is probably only partly correct. It is a consistent finding that lipids accumulate in insulin resistant muscle[3,20] and this is not a qualitative phenomenon (impaired mitochondrial respiration), but rather a quantitative phenomenon (decreased mitochondrial mass). The obvious question is therefore why the mitochondrial mass seems to be lower in the patients with type 2 diabetes? One explanation could be that the matching of the subjects is not optimal in the studies[12,18,21] where a lower mitochondrial content was reported. If the healthy control group and the patients with type 2 diabetes are carefully matched for physical activity level and maximal oxygen uptake, no differences exist in mitochondrial content, or intrinsic mitochondrial function[16]. Another question is the likelyhood of a marked decrease in mitochondrial content in the skeletal muscle of patients with type 2 diabetes. If a 30% decreased mitochondrial mass was indeed present in type 2 diabetes with a marked effect on respiratory capacity at rest (*ex vivo*), then one would expect that the in vivo exercise capacity would be severely impaired, because the mitochondrial respiratory rates increases more than tenfold with the transition from rest to exercise. Although there may be some exercise intolerance in patients with type 2 diabetes[22], most can be explained by altered oxygen uptake kinetics[23,24] on the background of impaired peripheral blood flow distribution/microvascular function. If a reduction in the mitochondrial content in the exercising skeletal muscle was a major limitation, then one would expect that skeletal muscle arterio-venous oxygen extraction would be impaired in type 2 diabetes. This is not the case[25].

It is well known that physical exercise increases skeletal muscle insulin sensitivity in patients with type 2 diabetes[26]. Furthermore, it has been reported that improvements in insulin sensitivity is accompanied by improvements in *in vivo* mitochondrial function[27]. It has been suggested that insulin resistant people may have an attenuated response to exercise training, compared with healthy control subjects[28]. Furthermore it has been reported that the response to an acute bout of exercise is attenuated in insulin-resistant compared with lean control subjects[29], when investigating genes coding for mitochondrial biogenesis (PGC-1α mRNA and protein abundance), which could explain the lack of a training effect in patients with type 2 diabetes in some studies[30-32]. It has been reported that different molecular signals in the skeletal muscle are responsible for the activation of mitochondrial biogenesis after exercise. These signals include elevated levels of cytosolic Ca^{2+}[33,34], AMP[33] and reactive oxygen species (ROS)[35]. All these studies are conducted in animals or cells, and have to our knowledge never been performed in patients with type 2 diabetes after an acute bout of exercise. An increased ROS production has also been linked to type 2 diabetes, but few human studies have actually investigated this and with conflicting results[8,10,36,37]. It has been reported in bovine aortic endothelial cells that hyperglycemia (30 mmol/L) increases ROS production[38].

This review will focus on adaptations in skeletal muscle mitochondria in patients with type 2 diabetes and healthy control participants after different exercise modalities (endurance, strength, high intensity training or a combination). Furthermore, we will attempt to clarify if the pharmacological treatment in patients with type 2 diabetes may blunt the training adaptations seen in non-diabetic people.

EFFECT OF MEDICATION ON EXERCISE ADAPTATIONS

Patients with type 2 diabetes are often treated with other medication to prevent high cholesterol and/or hypertension. In Denmark approximately 75% of all patients with diabetes are treated for hypertension, and approximately 64% are treated for hypercholesterolemia primarily with statins[39]. In Denmark approximately 90% of patients with type 2 diabetes are treated with metformin[39].

Antidiabetic agents

If a lifestyle intervention (diet and exercise) is not sufficient, metformin is the first drug of choice in the newly diagnosed patient with type 2 diabetes. Sulfonylurea may be added, and with poor glycemic control insulin treatment may be initiated. The adaptations to exercise are inadequately investigated when combined with these different medications. The mechanisms behind the glucose lowering effect of metformin is not known in detail, but a decrease in hepatic glucose production[40] and an

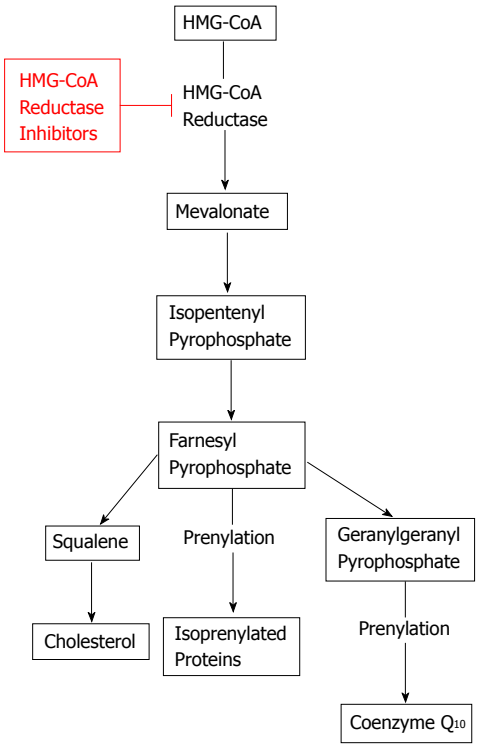

Figure 1 Effect of HMG-CoA reductase inhibitors (statins) on cholesterol and coenzyme Q10. HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A.

increase in glucose disposal in skeletal muscle *via* activation of AMPK[41] contributes to this. Metformin does not stimulate insulin secretion. In contrast, the hypoglycaemic effect of sulfonylureas is mediated *via* activation of the insulin producing beta cells[42], and these drugs have no direct effect on liver or skeletal muscle.

It has been suggested that the glucose lowering effect of metformin takes place *via* an inhibition of complex I in the electron transport chain in the mitochondria[43-45]. One study conducted in patients treated with metformin (2000 ± 200 mg/d) reported no effect on complex I in the electron transport chain[15]. A therapeutic dose of metformin of 1000 mg in humans corresponds to a plasma metformin concentration of approximately 0.1 mmol/L[46,47], and the peak metformin concentration in skeletal muscle is much lower than in the plasma[48]. In the studies were an inhibition was seen on complex I after metformin treatment, the concentrations used were high and supraphysiological[43-45].

It has been investigated whether metformin has an effect of exercise adaptations in young healthy subjects. One study measured maximal oxygen uptake in a double-blinded, placebo-controlled, cross-over study in healthy men and women and found a 2.7% reduction in maximal oxygen uptake after 7-9 d of treatment[49]. The authors suggest that this is unlikely to cause any individual impairment in exercise tolerance. Whether the same reduction is seen in patients with type 2 diabetes needs to be investigated. Even though 2.7% is not a major reduction, it could be argued that patients with type 2 diabetes would suffer more from this, due to a potential lower starting point. However, this finding was not confirmed in a similar study, where solely males participated[50]. Rosiglitazone (thiazolidinedione) is another antiglycemic agents, which has been reported to increase maximal oxygen uptake after 4 mo of treatment[51], the mechanisms behind the improvement is unknown.

A large proportion of patients diagnosed with type 2 diabetes have other co-morbidities, such as obesity, hypertension and dyslipidemia, *i.e.*, components of the metabolic syndrome. The pharmacological treatment of these may interfere with skeletal muscle and mitochondrial adapation to exercise training, and the literature regarding this issue will briefly be reviewed.

Lipid-lowering agents (statins)

Is has recently been reported that statins impairs the beneficial adaptations (increased maximal oxygen uptake and mitochondrial content) normally gained after a training intervention[52]. Different studies (longitudinal and cross-sectional) have reported an impaired mitochondrial function after statin therapy[53,54], which may compromise the OXPHOS capacity of the skeletal muscle. This would, in turn, further cause exercise intolerance. It has been suggested[54] that the culprit behind the impaired mitochondrial function, maybe a reduced coenzyme Q_{10} content in the skeletal muscle (Figure 1). It must be mentioned that not all studies have found a negative effect of statin therapy in combination with exercise[55]. In the study by Meex *et al*[55] many different statins were used, which could influence the result, since it is known that statins differs in lipophilicity[56], and thereby the ability to cross cell membranes. Is has also been reported that statins impaires complex I respiration in the electron transport chain[57]. Another group reported that simvastatin increased ROS production in human skeletal myotubes in combination with an impaired mitochondrial respiratory capacity[58]. To make it even more complex, it has been demonstrated that statins have opposite effects on mitochondria from cardiac and skeletal muscle[59]. Furthermore, studies have reported that statins have an effect (impairment or improvement) on insulin sensitivity (for review see[60]).

Antihypertensive agents

Diuretics, beta-blockers, calcium antagonists, ACE-inhibitors and angiotensin receptor blockers (ARBs) are all suitable for the initiation and maintenance of antihypertensive treatment, either as monotherapy or in combination therapy.

There are different kinds of β-blockers known as selective or nonselective. The selective can either block β_1 (cardiac) or β_2 (skeletal muscle) receptors, the nonselective ones blocks both receptors. The adaptations to exercise training can be influenced by using either kind[61]. Ades *et al*[62] investigated 10 wk of endurance training (4 times a week) in hypertensive patients, taking either metoprolol (β_1 selective β-adrenergic blocker), propranolol (β_1 nonselective β-adrenergic blocker) or placebo. They reported an improvement in maximal oxygen uptake

and an increase in mitochondrial content (succinyl dehydrogenase activity) in the placebo and the metoprolol group, whereas no improvements after training was seen with propranolol[62]. Another group investigated 6 wk of endurance training in healthy young subjects[63]. The subjects were randomized to either a selective (atenolol) or nonselective (nadolol) β-adrenergic blocker or placebo. Subjects receiving placebo improved maximal oxygen uptake to a higher extent than the two groups receiving medication, but all groups improved maximal oxygen uptake from baseline. Furthermore mitochondrial content increased in all three groups after training, but again the placebo-group improved to a higher extent[63]. Similar results were reported by Svedenhag *et al*[64] in young healthy subjects after 8 wk of endurance training.

Drexler *et al*[65] investigated the short- and long-term effect of ACE inhibition on patients with congestive heart failure at rest and during exercise. They reported an improvement in oxygen extraction of the working muscle after ACE inhibition, and they speculate that this could be due to an increased mitochondrial content, but unfortunately muscle biopsies were not obtained to elucidate this[65]. In addition, studies have investigated the effect of ACE inhibitors on insulin sensitivity and found divergent results, with either no effect[66] or an improvement[67].

It has previously been reported that Angiotensin Ⅱ receptor blockers (ARBs) have a positive effect on reactive oxygen species production and mitochondrial function in animals (for review see[68]). It has been reported that ARBs have different effects on glucose homeostasis in hypertensive patients with the metabolic syndrome[69]. Patients treated with telmisartan showed an improvement in HOMA-IR and HbA1c (surrogate measures of insulin sensitivity[70]), whereas patients treated with losartan showed no improvement[69]. Whether these improvements can be explained by improvements in mitochondrial function is at the moment impossible to say.

These results highlight the importance of controlling the medication when mitochondrial function and insulin sensitivity are measured before and after a training intervention. Otherwise the results obtained will be hard to explain. Furthermore, the interaction between the different drugs is also unknown, and would off course also be a confounding factor when results are interpretated.

MUSCULAR ADAPTATION TO DIFFERENT TRAINING MODALITIES IN PATIENTS WITH TYPE 2 DIABETES

It is well known that exercise interventions improve maximal oxygen uptake, mitochondrial content and insulin sensitivity in healthy subjects[71-75].

Different training modalities have been investigated in patients with type 2 diabetes and control subjects, to see if the training adaptation is similar in patients compared with control participants. Unfortunately many of the studies investigating the effect of exercise in patients with type 2 diabetes are lacking a healthy matched control group, which makes it impossible to compare the response between patients and control participants. Furthermore, the medication used is often not described in detail. In this review we primarily report the studies that have measured maximal oxygen uptake, mitochondrial function and insulin sensitivity (clamp, OGTT, HbA1c or fasting glucose and insulin concentrations).

Endurance training

Hey-Mogensen *et al*[8] investigated if 10 wk of endurance training affected mitochondrial function, maximal oxygen uptake and insulin sensitivity. The patients with type 2 diabetes in this study were treated with either metformin or sulfonylurea, other kinds of medication were not mentioned in the manuscript. A similar improvement in VO_{2max} was seen in patients (12%) and control participants (16%). Insulin sensitivity was significantly increased after training in both control participants (22%) and patients (13%). Mitochondrial OXPHOS capacity and intrinsic mitochondrial function was measured in isolated mitochondria, with no differences between patients and control subjects, except for the increased capacity to oxidize long chain fatty acids after training in patients with type 2 diabetes which was not apparent in the control participants. This finding is in contrast to the hypothesis about reduced ability to oxidize lipids in patients with type 2 diabetes[76,77], and therefore it indicates that impaired insulin sensitivity is not caused by a reduced mitochondrial capacity for lipid oxidation. Furthermore, CS activity also increased similarly in the groups. Interestingly no differences were seen in PGC-1α (mRNA) after training in either patients or control participants. PGC-1α is a major regulator for mitochondrial biogenesis[78]. Mitochondrial ROS production was similar in the two groups and did not change significantly with training. An increased UCP3 protein content was seen, but only in the control participants[8]. It has previously been suggested that UCP3 is acting as a protective mechanism against ROS production[79]. No difference was seen in intrinsic mitochondrial respiratory function between patients and control participants in this study (both before and after training)[8], this finding is contradictory to another study (cross-sectional) from the same group, where a lower intrinsic mitochondrial function was seen in patients with type 2 diabetes[4]. Another study investigated the effect of a combination of endurance and strength training (12 wk)[17] and similar to the study by Hey-Mogensen *et al*[8] no information is available in the manuscript regarding other kinds of medication except for the glucose lowering agents (metformin or sulfonylurea). An increased VO_{2max} was seen after training in the patients with type 2 diabetes, where only a tendency was seen in the control participants. An increased mitochondrial content (mtDNA) was seen after training in both groups, accompanied by a similar intrinsic mitochondrial function before and after training in both groups[17], indicating that mitochondrial OXPHOS capacity was increased to a similar extent

Table 1 Effect of endurance training on maximal oxygen uptake, mitochondrial function and insulin sensitivity

Ref.	Subjects	Training	Duration	VO_{2max}	Mito	IS
Mogensen *et al*[81]	T2DM and CON	ET	10 wk (2-3 times/wk)	↑ T2DM ↑ CON	↑ T2DM ↑ CON	↑ T2DM ↑ CON
Hey-Mogensen *et al*[8]	T2DM and CON	ET	10 wk (4-5 times/wk)	↑ T2DM ↑ CON	↑ T2DM ↑ CON	↑ T2DM ↑ CON
Phielix *et al*[17]	T2DM and CON	ET and ST	12 wk (3 times/wk)	↑ T2DM → CON	↑ T2DM ↑ CON	↑ T2DM → CON
Meex *et al*[27]	T2DM and CON	ET and ST	12 wk (3 times/wk)	↑ T2DM ↑ CON	↑ T2DM ↑ CON	↑ T2DM → CON
Shaw *et al*[83]	T2DM	ET	6 mo (3 times/wk)	↑ T2DM	↑ T2DM	→ T2DM
Sparks *et al*[31]	T2DM and	ET	9 mo (150 min/wk)	→ T2DM	→ T2DM	→ T2DM
Bajpeyi *et al*[32]	T2DM and CON (L and O)	ET	10 d (every day)	ND	→ T2DM → CON (L and O)	ND
Nielsen *et al*[84]	T2DM and CON	ET	10 wk (4-5 times/wk)	↑ T2DM ↑ CON	↑ T2DM ↑ CON	↑ T2DM ↑ CON

CON: Control participants; ET: Endurance training; IS: Insulin sensitivity [or surrogate measures of insulin sensitivity (HbA1c, HOMA)]; L: Lean; O: Obese; VO_{2max}: Maximal oxygen uptake; Mito: Mitochondrial function (mitochondrial respiratory capacity, mitochondrial content); ND: Not determined; T2DM: Patients with type 2 diabetes.

in both groups (data not shown in the manuscript). It has recently been reported that mtDNA is not a good marker for mitochondrial content, at least not in healthy young subjects[80]. Phielix *et al*[5] has previously reported impaired intrinsic mitochondrial function in patients with type 2 diabetes, a finding that contradicts their own finding from 2010[17]. Meex *et al*[27] used the same training protocol as Phielix *et al*[17] with a combination of endurance and strength training for 12 wk. Again only glucose lowering medication is mentioned in the manuscript and thus not the pharmacological specification. Mitochondrial function was measured by magnetic resonance spectroscopy, and a difference was seen before training between the two groups with no difference present after training. Mitochondrial content was measured as complex I -V protein content (average of the complexes), both groups increased the average of the five complexes, but the increase tended to be more pronounced in the patients with type 2 diabetes. Maximal oxygen uptake increased significantly with training in both groups, whereas only patients with type 2 diabetes improved insulin sensitivity (clamp) after training[27]. Mogensen *et al*[81] conducted another study in which the effect of endurance training on skeletal muscle was studied. Again they showed a similar response in regard to maximal oxygen uptake, insulin sensitivity and mitochondrial content CS activity, where both groups improved in all parameters after training[81]. Nine months of aerobic training (150 min/wk at 50%-80% of VO_{2peak}) in patients with type 2 diabetes did surprisingly not improve either mitochondrial content, maximal oxygen uptake or insulin sensitivity, but an increased lipid oxidation was present after training[31]. The patient's medical records were not included in the manuscript, and no healthy control group was included. So the lack of improvement in mitochondrial content after 9 mo of aerobic training could be explained by the medication used (statins most likely). The study was an ancillary study to the HART-D study where the patients medical records are included, and a high percentage of the patients were in statin therapy[82]. Shaw *et al*[83] investigated 6 mo of endurance exercise (corresponding to approximately 77% of VO_{2peak}), they found an increased maximal oxygen uptake and mitochondrial content (COX activity), but no difference in insulin sensitivity. They did not report the medication used, but states that medication was stopped three days prior to the test days, indicating that the patients were on medication during the training period, and in addition an appropriate control group was not investigated. Another group investigated lean, obese and patients with type 2 diabetes before and after 10 d of 60 min exercise at 70% of VO_{2peak}[32]. No differences were seen in muscle oxidative capacity between groups before and after training, which is quite intriguing taking into consideration that the lean subjects had a higher maximal oxygen uptake (approximately 50%) compared with the two other groups. Insulin sensitivity was unfortunately not measured[32]. Mitochondrial volume (by TEM) was investigated after 10 wk of endurance training (approximately 70% of VO_{2max}) and a similar increase in mitochondrial volume was seen in patients with type 2 diabetes and control participants, accompanied by improvements in maximal oxygen uptake and insulin sensitivity (clamp)[84]. Table 1 gives an overview over the published literature in regard to endurance training.

High intensity training

The last five to ten years a renewed interest has been directed towards a different training method, where high intensity training is performed for shorter durations. It has been reported that high intensity training (HIT) leads to similar metabolic adaptations compared to regular endurance training when it comes to improvement in maximal oxygen uptake and increase in mitochondrial content in healthy human skeletal muscle[72,73]. This has not been investigated thoroughly in patients with type 2 diabetes.

Two weeks of HIT has been reported to increase mitochondrial content (CS activity) and improve 24 h blood glucose profile (measured 48-72 h after last training

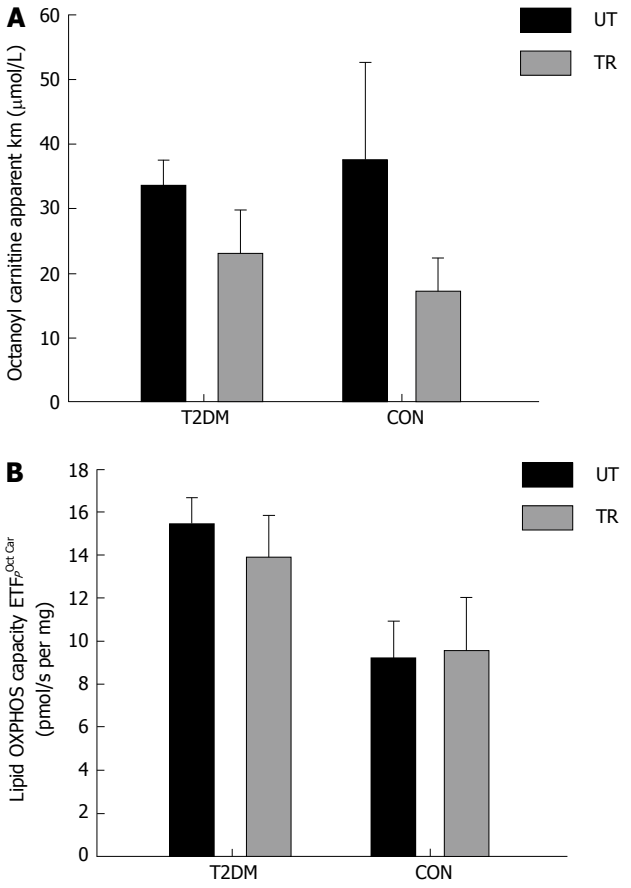

Figure 2 Patients with type 2 diabetes (*n* = 5) and healthy control subjects (*n* = 5) performed eight sessions of one-legged high intensity training in two weeks. Each session consisted of ten one-minute exercise bouts at 60% of one-legged maximal oxygen uptake and > 80% of maximal heart rate, interspersed by one min rest. After completion of the training muscle biopsies (vastus lateralis) were obtained from the untrained (black bars) and the trained (grey bars) leg. The measurement mitochondrial OXPHOS capacity and substrate sensitivity was performed with malate, ADP and octanoyl carnitine (titration: 5-2000 μmol/L). A: Apparent Michaelis Menten constant Km for octanoyl carnitine; B: Maximal OXPHOS capacity with the mentioned substrates. T2DM: Type 2 diabetes; CON: Control subjects; UT: Untrained; TR: Trained.

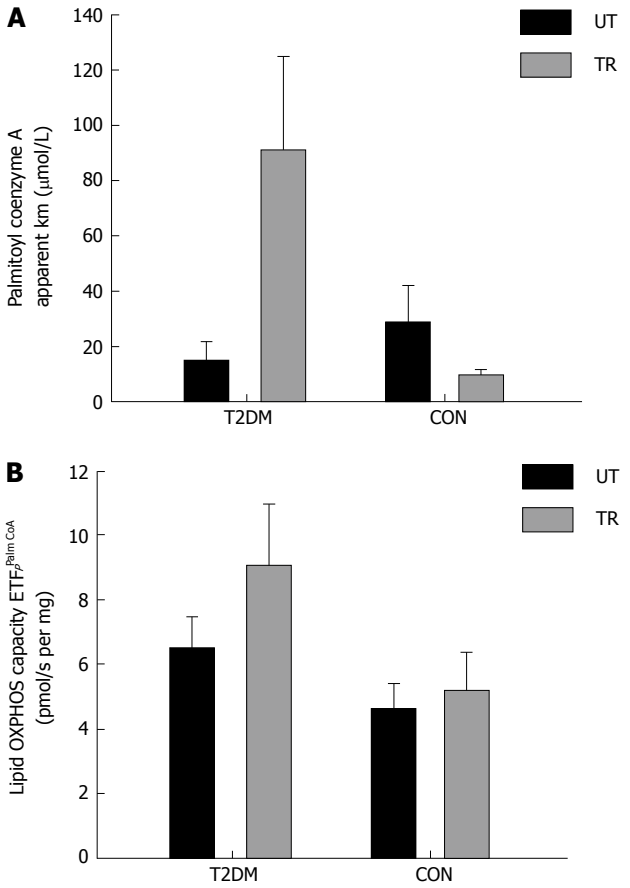

Figure 3 Patients with type 2 diabetes (*n* = 5) and healthy control subjects (*n* = 3) performed eight sessions of one-legged high intensity training in two weeks. Each session consisted of ten one-minute exercise bouts at 60% of one-legged maximal oxygen uptake and > 80% of maximal heart rate, interspersed by one min rest. After completion of the training muscle biopsies (vastus lateralis) were obtained from the untrained (black bars) and the trained (grey bars) leg. The measurement mitochondrial OXPHOS capacity and substrate sensitivity was performed with malate, ADP and palmitoyl coenzyme A (titration: 5-100 μmol/L). A: Apparent Michaelis Menten constant Km for palmitoyl coenzyme A; B: Maximal OXPHOS capacity with the mentioned substrates. T2DM: Type 2 diabetes; CON: Control subjects; UT: Untrained; TR: Trained.

bout)[85] in patients with type 2 diabetes. Unfortunately no control group was included by Little and colleagues[85], but in another study a similar improvement in CS activity was observed in overweight women using the same training protocol[86].

We have recently investigated mitochondrial substrate sensitivity in patients with type 2 diabetes and control participants after two weeks (eight training sessions) of one legged HIT [pilot study (type 2 diabetes; *n* = 5-7; control subjects; *n* = 3-5)]. Each training session consisted of ten one minute bouts of high intense one-legged bicycle exercise interspersed with one minute recovery. The training load corresponded to minimum 60% of the maximal workload obtained during a one-legged maximal oxygen uptake test. Due to the low number of subjects investigated we did not perform any statistical analysis on the dataset. We used high resolution respirometry and measured the mitochondrial ability to use either octanoyl carnitine (medium chain fatty acid), palmitoyl coenzyme A (long chain fatty acid, using carnitine palmitoyltransfer ase I (CPT I) to enter the mitochondrion) and palmitoyl carnitine (long chain fatty acid, using CPT Ⅱ to enter the mitochondrion). The results from the pilot study (respirometric measurements) are provided in Figures 2-4. The method we used has been described previously[12,16]. No differences were seen in mitochondrial substrate sensitivity for octanoyl carnitine between the groups and both groups increased their sensitivity for octanoyl carnitine in the trained leg (Figure 2A). It has been reported previously that no differences are present in mitochondrial subtrate sensitivity with octanoyl carnitine between patients with type 2 diabetes and obese participants[16]. No effect was seen after training in regard to maximal mitochondrial oxidative capacity with octanoyl carnitine as a substrate, but is seems as the patients with type 2 diabetes have a higher capacity to oxidize medium chain fatty acids (Figure 2B). Mitochondrial substrate sensitivity for palmitoyl coenzyme A (Figure 3A) and palmitoyl

Table 2 Effect of strength training on maximal oxygen uptake, mitochondrial function and insulin sensitivity

Ref.	Subjects	Training	Duration	VO_{2max}	Mito	IS
Holten *et al*[30]		ST (one leg)	6 wk (3 times/wk)	ND	→ T2DM ↑ CON	↑ T2DM ↑ CON
Sparks *et al*[31]	T2DM	ST	9 mo (3 times/wk)	→ T2DM	↑ T2DM	→ T2DM

CON: Control participants; IS: Insulin sensitivity [or surrogate measures of insulin sensitivity (HbA1c, HOMA)]; VO_{2max}: Maximal oxygen uptake; Mito: Mitochondrial function (mitochondrial respiratory capacity, mitochondrial content); ND: Not determined; ST: Strength training; T2DM: Patients with type 2 diabetes.

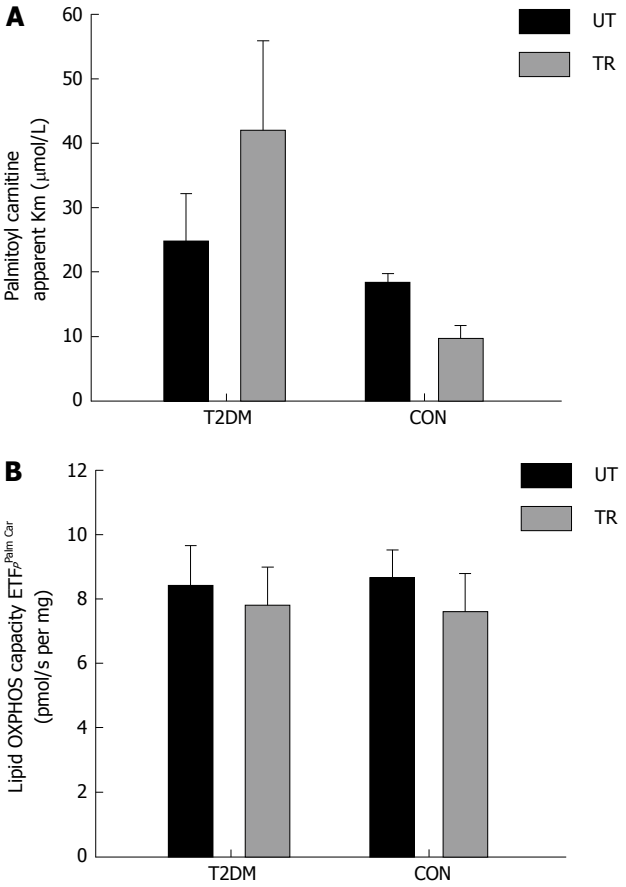

Figure 4 Patients with type 2 diabetes (n = 7) and healthy control subjects (n = 5) performed eight sessions of one-legged high intensity training in two weeks. Each session consisted of ten one-minute exercise bouts at 60% of one-legged maximal oxygen uptake and > 80% of maximal heart rate, interspersed by one min rest. After completion of the training muscle biopsies (vastus lateralis) were obtained from the untrained (black bars) and the trained (grey bars) leg. The measurement of mitochondrial OXPHOS capacity and substrate sensitivity was performed with malate, ADP and palmitoyl carnitine (titration: 5-200 μmol/L). A: Apparent Michaelis Menten constant Km for palmitoyl carnitine; B: Maximal OXPHOS capacity with the mentioned substrates. T2DM: Type 2 diabetes; CON: Control subjects; UT: Untrained; TR: Trained.

carnitine (Figure 4A) showed the same tendency where patients with type 2 diabetes had a decreased (apparent Km increased) sensitivity for long chain fatty acid (palmitoyl coenzyme A and carnitine) and control participants an increased (apparent K_m decreased) sensitivity after training. No major differences were seen in maximal mitochondrial oxidative capacity with palmitoyl coenzyme A (Figure 3B) or palmitoyl carnitine (Figure 4B) between the groups and after the training intervention. From these pilot data, it seems as if no differences are present between patients and control participants in regard to maximal mitochondrial oxidative capacity with fatty acids as substrate either at baseline or after the training intervention. The improved sensitivity for CPT Ⅰ and CPT Ⅱ in the control participants, could be explained by an increased activity of CPT Ⅰ (and maybe CPT Ⅱ) which have been reported previously[87]. Why patients with type 2 diabetes show an opposite adaptation is difficult to explain, but it has been reported that CPT Ⅰ activity is reduced in skeletal muscle from obese compared to lean participants[88]. To our knowledge the effect of training on CPT Ⅰ and Ⅱ activity has never been investigated in patients with type 2 diabetes, and it is thus impossible to say whether this can explain our results. Little *et al*[85] gives an overview over the published literature in regard to high intensity training.

Strength training

Is has been suggested previously that strength training represents an attractive training modality, due to the fact that many patients with type 2 diabetes are obese and have difficulties performing endurance exercise.

Few studies have been performed where adaptations in skeletal muscle have been investigated. Holten *et al*[30] investigated 6 wk (3 times per week) of leg strength training (one leg, other leg served as control) and found improvement in the trained leg in both groups regarding insulin sensitivity (clamp technique). Maximal oxygen uptake was not measured, but mitochondrial content (by CS activity) showed no difference between the legs in the patients but an increase was observed in the control participants. Nine months of resistance training increased mitochondrial content in patients with type 2 diabetes, but no difference was seen in maximal oxygen uptake and HbA1c[31]. This study contradicts the findings by Holten *et al*[30], and this may be due to a difference in duration and application of different methods to evaluate insulin sensitivity. Table 2 gives an overview over the published literature in regard to strength training.

CONCLUSION

From the literature currently available it is difficult to recommend a training intervention to patients with type 2 diabetes where success is well documented when it comes to improvement in mitochondrial function. The problem with many of the studies available is that medicine usage

is not reported, and therefore potential significant medication effects on the outcome can not be excluded, when adaptations to physical activity are investigated. Furthermore, many of the studies lack a real control group, making it impossible to determine if adaptations are the same in patients and control participants.

The literature is at current lacking well conducted controlled longitudinal studies investigating the effect of exercise on mitochondrial function, where medication is controlled and an appropriate control group is included. These studies are difficult to conduct given the ethical problem in how you control the medication without compromising and disrupting the health of the patients. One approach could be to recruite newly diagnosed patients, where medication is not started yet. A study like this needs to be conducted in the future where mitochondrial function is investigated.

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P- Reviewer: Davison GW **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Genetic polymorphisms of cytokine genes in type 2 diabetes mellitus

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Author contributions: Both the authors contributed equally to the manuscript.
Supported by Agencies *viz* Department of Biotechnology (DBT), Indian Council of Medical Research (ICMR), Department of Science and Technology (DST) and Centre of Excellence (COE), UP Government, India for generous grants to our laboratory for diabetes research
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Received: November 28, 2013 Revised: May 26, 2014
Accepted: June 14, 2014
Published online: August 15, 2014

Abstract

Diabetes mellitus is a combined metabolic disorder which includes hyperglycemia, dyslipidemia, stroke and several other complications. Various groups all over the world are relentlessly working out the possible role of a vast number of genes associated with type 2 diabetes (T2DM). Inflammation is an important outcome of any kind of imbalance in the body and is therefore an indicator of several diseases, including T2DM. Various ethnic populations around the world show different levels of variations in single nucleotide polymorphisms (SNPs). The present review was undertaken to explore the association of cytokine gene polymorphisms with T2DM in populations of different ethnicities. This will lead to the understanding of the role of cytokine genes in T2DM risk and development. Association studies of genotypes of SNPs present in cytokine genes will help to identify risk haplotype(s) for disease susceptibility by developing prognostic markers and alter treatment strategies for T2DM and related complications. This will enable individuals at risk to take prior precautionary measures and avoid or delay the onset of the disease. Future challenges will be to understand the genotypic interactions between SNPs in one cytokine gene or several genes at different loci and study their association with T2DM.

Key words: Type 2 diabetes; Cytokines; Single nucleotide polymorphisms; Disease susceptibility; Association studies

Core tip: Diabetes is the third most widespread disease after heart disease and cancer. Cytokines are mediators of inflammation, namely interleukins (IL)-1β, -1Ra, -18, -4, -6, -10, tumor necrosis factor-α and adiponectin, which cause immune responses in disease pathogenesis, including type 2 diabetes. In the present study, the association of cytokine gene polymorphisms in different ethnic populations is reviewed. Such single nucleotide polymorphism analyses and association studies in different populations will benefit individuals belonging to a particular group.

Original sources: Banerjee M, Saxena M. Genetic polymorphisms of cytokine genes in type 2 diabetes mellitus. *World J Diabetes* 2014; 5(4): 493-504 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/493.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.493

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a group of metabolic disorders characterized by high blood sugar levels, which results from defects in insulin secretion or action or both, leading to complications[1]. Diabetes mellitus has now

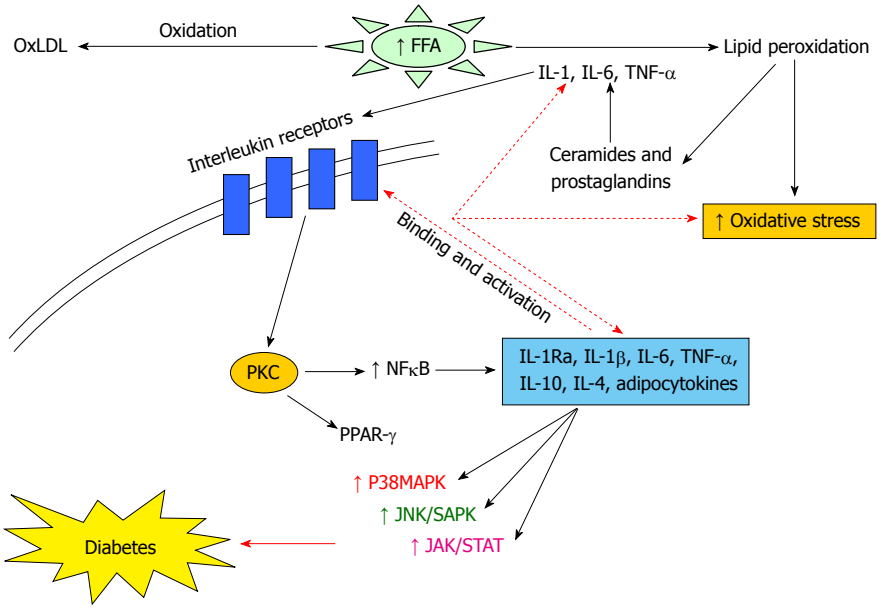

Figure 1 A schematic diagram showing the involvement of various cytokines in diabetes[3]. IL: Interleukin; TNF: Tumor necrosis factor.

been associated with the development of a long term organ disease. T2DM has changed from a mild disorder of old age to a serious cause of morbidity and mortality in young and middle-aged people. The Diabetes Atlas estimates have shown that 371 million people suffer from diabetes worldwide, with India alone having 63.0 million affected individuals and the number is expected to rise to 101.0 million by 2030[2-4]. This alarming figure has instigated several workers worldwide to undertake genetic studies and contribute to the understanding and early detection of the disease.

A predisposition to T2DM or "Adult Onset Diabetes" is probably inherited as an autosomal recessive trait[5]. T2DM is treated initially by diet control, either alone or in combination with orally administered anti-diabetic drugs. It is described as a syndrome on the basis of clustering of many abnormalities, like resistance to insulin-stimulated glucose uptake, hyperinsulinemia, hyperglycemia, increased very low density lipoprotein (VLDL), increased triglycerides, decreased high density lipoproteins (HDL) cholesterol, high blood pressure, micro albuminuria, hyperuricemia, fibrinolytic and coagulation abnormalities, *etc*[3].

Evidence has shown that T2DM is associated with chronic inflammation that can be attributed to dysregulation of the innate immune system and this is a potential link between metabolic syndrome, diabetes and atherosclerosis[6]. A large and diverse family of small, low molecular weight cell signaling proteins mediating complex interaction are called "cytokines", which include interleukins and interferons[7] secreted by white blood cells and various other cells in response to a number of stimuli. The cytokines and their receptors exhibit a very high affinity for each other. Another subgroup of low molecular weight cytokines called chemokines affect leukocyte behavior. Cytokines are of two types, namely pro-inflammatory [*e.g.*, interleukins (IL)-1, -6, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β] and anti-inflammatory (*e.g.*, IL-1Ra, -4, -10, -13), which function opposite to each other. The release of adipocytokines by adipocytes, such as leptin, resistin, adiponectin and visfatin, as well as some of the classical inflammatory cytokines like TNF-α, IL-6, MCP-1 (CCL-2) *etc*., help to achieve this. Studies have shown that it is the fat tissue that exerts the endocrine and immune functions. Macrophages and T cells are found in abundance in adipose tissue which develops into an organized immune organ[8]. Inflammation resulting from an imbalance between pro- and anti-inflammatory cytokines leads to T2DM and its complications (Figure 1).

Mediators of inflammation, such as IL-1β, -1Ra, -18, -4, -6, -10, TNF-α and adiponectin (ADIPOQ), have been proposed to be involved in causing T2DM. Elevated blood levels of certain acute phase markers such as IL-6 can characterize the immune response[9], while IL-1 regulates the basic metabolic rate, blood glucose levels, blood pressure, iron metabolism and bone remodeling. Adiponectin levels and its gene variants have also been confirmed to be associated with increased risk of T2DM[10]. To date, more than 1240 gene loci are associated with diabetes in humans[3]. The susceptibility to complex forms of T2DM is associated with frequent polymorphisms that influence the expression of genes belonging to the same or different causal pathways[7]. It is important to understand the nature and actions of these adipocytokines in order to find their association with diseases like T2DM, atherosclerosis, other metabolic and vascular diseases (Figure 2). Studies have reported that Asian Indians are a unique population for carrying out genetic studies due to their greater susceptibility to T2DM and increased insulin resistance[11,12]. This review is an attempt to put together certain important cytokine gene polymorphisms and their association with T2DM in

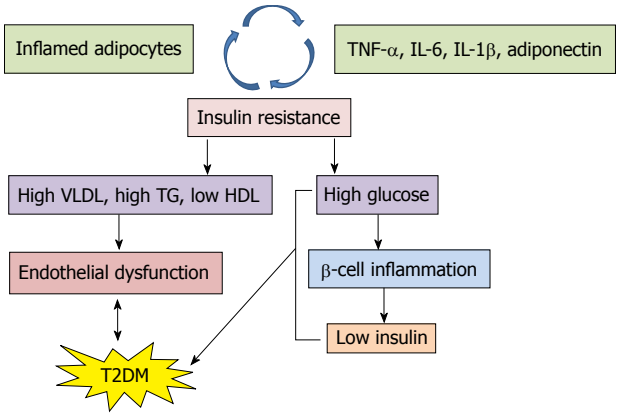

Figure 2 A schematic diagram showing the metabolic defects and biochemical effects of cytokines leading to type 2 diabetes. T2DM: Type 2 diabetes; IL: Interleukin; TNF: Tumor necrosis factor.

different populations around the world.

CYTOKINE GENE POLYMORPHISMS AND T2DM

Certain chemokines/cytokines, like IL-1β, -1Ra, -18, -4, -6, -10, TNF-α, *etc.*, and some members of the adipocytokine family, namely adiponectin, leptin and resistin, are important mediators in inflammation/disease and glucose metabolism and may be involved in the pathogenesis of T2DM. They can be used as biological markers for diabetes and are related to obesity and hypertension. The single nucleotide polymorphisms (SNPs) present in the regulatory regions of cytokine genes often have an impact on their expression levels and can be disease modifiers. The degree of inflammation is controlled, thereby leading to the progression of various immunological diseases, including T2DM[13-20]. The polymorphisms in cytokine genes lead to interindividual differences in their production, leading to variations in immune responses[21].

IL-1α, -1β and -Ra

The IL-1 family consists of two pro- and one anti-inflammatory cytokines, namely 1α, 1β and the IL-1 receptor antagonist (IL-1Ra), respectively. While IL-1α and -1β enhance inflammation and host defense, IL-Ra counteracts their function. A variety of cell types like monocytes/macrophages and keratinocytes are known to produce these cytokines. All three secreted glycoproteins bind to IL-1 receptors[22].

The *IL-1* genes (*IL-1α*, *-β* and -Ra) are located on chromosome 2q12-21. All *IL-1* genes are polymorphic and several are associated with inflammation and disease conditions[7,23]. "Autocrine apoptosis" results from prolonged exposure of human islets to high glucose which triggers IL-1β production, leading to activation of nuclear factors and upregulation of Fas signaling[24]. IL-1β and IL-1Ra play important roles in tissue remodeling, are potent mediators of chronic inflammation[25] and are therefore implicated in the pathogenesis of T2DM and associated complications[7]. The *IL-1* gene variants studied in various groups are shown in Table 1.

Table 1 Variants of interleukin-1 gene cluster (interleukin-1α, interleukin-1β, interleukin-1Ra, interleukin-18) and their association with type 2 diabetes in different populations

Gene	Variants (SNPs)	Population-Ethnic group	Association	Ref.
IL-1α	-889		NS	[26]
IL-1β	3954			
IL-1β	-511			
IL-1Ra	VNTR			
IL-1α	3'UTR	Caucasians and African Americans	S	[27]
IL-1	C-889T	East Indian	S	[28]
IL-1β	C-511T			
IL-1β	C3953T			
IL-1α			S	[29]
IL-Ra	VNTR			
IL-1β	C3954T		S	[30]
IL-1β	-511	North Indian	S	[31]
IL-1Ra	VNTR			
IL-1β	C-511T		S	[32]
IL-1Ra	VNTR			
IL-1β	C-511T	Korean	S	[33]
IL-1Ra	VNTR			
IL-1Ra	VNTR		NS	[29]
IL-1Ra	VNTR		S	[34]
IL-1Ra	VNTR	North Indian	S	[17]
IL-1Ra	VNTR	Caucasians	NS	[35]
IL-1Ra	VNTR		S	[36]
IL-1RI	PstI, HinfI, AluI (promoter region) PstI (exon 1B region)	Dalmatian population of South Croatia	S	[37]
IL-18	+183 A/G	Norwegian	S	[38]
	-137 G/C		NS	
	-607 C/A		NS	
	-607 C/A	Chinese	S	[39]
	BCO2	European	S	[40]
	rs2250417	European	NS	[41]
	5 SNPs	European	S	[42]

UTR: Untranslated region; VNTR: Variable number of tandem repeats; S: Significant; NS: Nonsignificant; IL-1: Interleukin-1; SNPs: Single nucleotide polymorphisms.

IL-18

IL-18, a unique IL-1 family cytokine is expressed in macrophages, keratinocytes, osteoblasts, synovial fibroblasts, dendritic, Kupffer, adrenal cortex, intestinal epithelial and microglial cells[43-50]. IL-18 shares structural homology with IL-1β. It is produced as a 24-kDa inactive precursor, Pro-IL-18, which is cleaved by IL-1β-converting enzyme (ICE; caspase-1) to a mature 18-kDa molecule[51]. The extracellular binding of IL-18 is mediated by IL-18R, a heterodimer complex containing α chain (IL-1Rrp) and β chain (AcPL)[52-54].

Insulin-producing islet β-cells secrete IL-18 and induce IFNγ in T cells[55]. IL-18 is highly expressed in atherosclerotic plaques with a role in plaque destabilization[56]. Elevated levels of plasma IL-18 were reported in T2DM patients and children[57-59]. However, obesity and insulin resistance showed no correlation with IL-18

Table 2 Variants of interleukin-4 gene and their association with type 2 diabetes in different populations

Gene variants (SNPs)	Disease	Population-Ethnic groups	Association	Ref.
-590 C/T	T2DM	Iranian	S	[64]
-589 C/T	T2DM	Chinese	S	[65]
-34 C/T	T2DM			
VNTR	T2DM	North Indian	S	[17]

VNTR: Variable number of tandem repeats; S: Significant; T2DM: Type 2 diabetes; SNPs: Single nucleotide polymorphisms.

plasma level[60]. The *IL-18* gene in humans is located on chromosome 11q22.2-22.3, where a diabetes susceptibility locus, Idd2, resides[61]. Studies reporting *IL-18* gene polymorphisms are shown in Table 1.

IL-4

One of the hematopoietic cytokines, IL-4 regulates key events during Th2-dominated immune response and also stimulates T cells, leading to the production of other cytokines. It causes β-cell isotype switching from IgM to IgE and stimulates IgE production in allergic sensitization. IgE stimulation during allergic reactions and infections is the natural defense mechanism. It also plays a crucial role in the pathophysiology of T2DM[62]. The heterodimerization of high-affinity transmembrane receptor α-chain (IL-4Rα) is mediated by IL-4 in a sequential cascade. Several candidate genes have been identified, including the gene for *IL-4Ra* which is situated on chromosome 16p and is known to contain a number of polymorphisms. IL-1Ra and IL-4 are major anti-inflammatory cytokines[63] and have been proposed to be involved in events causing T2DM. The IL-4Ra subunit forms part of the signalling complex for IL-4. In humans, the gene for *IL-4* maps to chromosome 5q31. The polymorphisms in *IL-4* gene and their relationship with T2DM have been studied by various groups (Table 2).

IL-6

IL-6 is secreted by immune cells, adipose tissue and muscles and is able to accelerate or inhibit the inflammatory processes[66,67]. The direct affect of IL-6 may be on glucose homeostasis and metabolism or it might act indirectly by action on adipocytes, pancreatic β-cells, *etc*[68]. In humans, the gene for *IL-6* maps to chromosome 7p15-p21. *IL-6* mRNA expression and insulin resistance were found to have a significant correlation[69] and increased plasma IL-6 levels with higher risk of T2DM[6,70,71], making it an appealing candidate gene. One of the common polymorphisms in the *IL-6* gene promoter (C-174G) was found to regulate transcription in response to inflammatory stimuli, such as lipopolysaccharides or IL-1[72-74]. IL-6 promoter SNPs were considered as risk factors for T2DM development, as reported by other groups[75,76] (Table 3).

IL-10

IL-10 is also a Th2 mediated cytokine that downregulates inflammatory responses of pro-inflammatory cytokines[102]. The serum concentrations of TC, LDL, TGL, glucose and HbA1c gradually decreases and HDL increases with an increase in IL-10 production. These observations implied that low IL-10 production was associated with hyperglycemia and T2DM[68,103]. IL-10 promotes the proliferation and differentiation of B-lymphocytes by stimulating antibody production[104]. The *IL-10* gene is located on chromosome 1q31-q32 and several variants have been identified in its promoter region[105-106]. The presence of IL-10 is protective against T2DM and

Table 3 Variants of Interleukin-6 gene and their association with type 2 diabetes and related complications in different populations

Gene variants (SNPs)	Diseases	Population-Ethnic groups	Association	Ref.
-174 G/C	T2DM and OGTT	Brazilian	S	[77]
	T2DM and IR	American	S	[78]
	T2DM and obesity	Polish	S	[79]
	T2DM and obesity	Mexican	NS	[80]
	T2DM	Indian	S	[81]
	T2DM	Finnish	NS	[82]
	T2DM and Obesity	Tunisian	S	[83]
	T2DM	Caucasian	S	[84]
	T2DM	German	S	[85]
	DM, micro-, macrovascular complications	Australian	NS	[29]
	-do-	German	NS	[86]
	T2DM and IR	Italian	S	[87]
	T2DM	KORA Survey	S	[88]
	T2DM	Framingham Heart Study	S	[89]
	T2DM	KORA Survey	S	[90]
	T2DM	Taiwanese	S	[91]
	T2DM	Nutrition-Potsdam cohort	S	[92]
	T2DM	Finnish	S	[93]
	T2DM	Native Americans, Spanish, Caucasians	S	[75]
	T2DM and IR	Spanish	S	[94]
	T2DM and PAD	Italian	S	[95]
	T2DM	KORA Survey	S	[76]
	DM and Periodontitis	Chinese	S	[96]
	T2DM and Endothelial Dysfunction	Chinese	S	[97]
	T2DM	21 studies	S	[71]
-174 G/C -597 A/G	T2DM	Boston	NS	[98]
GWS (18 SNPs)	T2DM	Canadian	S with Fasting	[99]
PREDIAN study	DN	Spanish	S	[100]
Five tagging SNPs	T2DM and Impaired Renal Function	Singaporean	S	[101]

S: Significant; NS: Non-significant; T2DM: Type 2 diabetes; PAD: Peripheral arterial disease; SNPs: Single nucleotide polymorphisms; OGTT: Oral glucose tolerance test; DM: Diabetes mellitus; IR: Insulin resistance; DN: Diabetic nephropathy.

Table 4 Variants of interleukin-10 gene and their association with type 2 diabetes and related complications in different populations

Gene variants (SNPs)	Diseases	Population-Ethnic groups	Association	Ref.
-592 A/C	T2DM	Iranian	NS	[108]
	T2DM	Chinese	NS	[109]
	T2DM	North Indian	S	[4]
-1082 G/A	proliferative diabetic retinopathy	Indian	S	[110]
	T2DM	South Indian	S	[111]
-1082 G/A -819 C/T -592 C/A	T2DM	Caucasian Italian	S	[112]
-1082 G/A	T2DM	Turkish	NS	[113]
-1082 G/A -819 C/T -592 C/A	T2DM	Greek	NS	[106]
-592 A/C -819 C/T	T2DM	Taiwanese	NS	[107]
-592 A/C	T2DM	Taiwanese	S	[114]
-1087 G/A -824 C/T -597 C/A	T2DM	Italian	S	[115]
-592 A/C	T2DM	Tunisian	S	[18]

S: Significant; NS: Non-significant; T2DM: Type 2 diabetes; SNPs: Single nucleotide polymorphisms.

Table 5 Variants of tumor necrosis factor-α gene and their association with type 2 diabetes and related complications in different populations

Gene variation (SNPs)	Diseases	Population-Ethnic groups	Association	Ref.
G-308A	T2DM	Tarragona	S	[120]
	T2DM	Taiwanese	S	[121]
	T2DM	Croatian Caucasians	S	[122]
	T2DM and peridontitis	Chinese	S	[123]
	T2DM, MS and Obesity	Indian	S	[124]
	T2DM	Mexican	S	[125]
	Glucose metabolism	Brazilian	S	[126]
	T2DM	Japanese	NS	[127]
	T2DM	Mexican	NS	[128]
	T2DM	Chinese	NS	[129]
	T2DM	Greek	NS	[130]
	atherosclerotic diabetic	Hungarian	S	[131]
	T2DM	Indian	S	[81]
	T2DM	United Kingdom/ Irish	NS	[132]
	T2DM	Finnish	S	[82]
sTNFR1 and sTNFR2	Glucose metabolism	Hungarian	NS	[133]
C-857T	IR and T2DM	Japanese	S	[134]

S: Significant; NS: Non-significant; T2DM: Type 2 diabetes; SNPs: Single nucleotide polymorphisms; MS: Metabolic syndrome; TNFR1: Tumor necrosis factor receptor 1.

inflammation due to its humoral immunity responses and prevention of pancreatic beta cell destruction[4,107]. The association of *IL-10* gene polymorphisms is shown in Table 4.

TNF-α

TNF-α is released by monocytes/macrophages and has an initial role in β-cell damage of the islets. It is reported that TNF-α is a possible mediator of insulin resistance and diabetes since it decreases the tyrosine kinase activity[116]. Furthermore, TNF-α inhibits insulin signaling[117] and impairs its secretion[118]. TNF-α interacts with IL-6, regulating its expression and downregulating itself[73]. In humans, the gene for *TNF-α* maps to chromosome 6p21. 3. One of the SNPs in *TNF-α* gene showed a two-fold increase in transcriptional activity[119,120]. Various groups showed an association of *TNF-α* SNPs with T2DM (Table 5).

Adiponectin

An endocrine effect leading to the clinical expression of T2DM and cardiovascular disease was attributed to the cytokines secreted by adipocytes[135,136]. Since the role of classical cytokines and adipocytokines in metabolic syndrome and associated disease conditions came to light, several workers have shown the role of activated innate immunity in the pathogenesis of T2DM[70,137]. Adiponectin levels in the plasma remain constant throughout the day and are not affected by food intake, unlike insulin and leptin.

Adipocytes secrete a plethora of cytokines, including adiponectin, resistin, leptin, IL-6, TNF-α, visfatin, RBP4, as well as free fatty acids, which alter insulin action and hepatic glucose production[138-140]. Adiponectin is a serum protein produced and secreted exclusively by adipose tissues, also known as adipocytes complement-related protein of 30 KDa (147 amino acids) (Acrp30). It is involved in the homeostatic control of circulating glucose and lipid levels[141]. Reduced adiponectin levels are documented in obese, insulin resistant and T2DM patients[116]. Adiponectin regulates glucose/lipid homeostasis *via* phosphorylation and activation of adenosine monophosphate activated protein kinase[142,143]. Another important function of adiponectin is to prevent the atherosclerotic vascular damage by suppressing interaction of monocytes/endothelial cells and adhesion molecules[144,145]. Therefore, high adiponectin levels are associated with reduced risk of T2DM[70]. In humans, the gene for *ADIPOQ* maps to chromosome 3q27. The SNPs in ADIPOQ studied by other researchers are shown in Table 6.

CONCLUSION

The greater tendency to diabetes in Indians may result from some genetic factors in addition to environmental and dietary factors. It is reported that the severity of diabetes (T2DM) in patients, from chronic to newly diagnosed, is related to certain biochemical and pathological examinations. The risk factors include lipid metabolism abnormalities (VLDL, HDL, LDL, TGA *etc.*) and re-

Table 6 Variants of adiponectin gene and their association with type 2 diabetes and related complications in different populations

Gene variants (SNPs)	Diseases	Population-Ethnic groups	Association	Ref.
+45 G/T	Obesity	Iranians	NS	[146]
	T2DM	Malaysian	S	[147]
	T2DM	Greek	NS	[148]
	MS	Chinese	S	[149]
	T2DM	Japanese	NS	[150]
	T2DM	Chinese	S	[151]
	Non-T2DM	Caucasian Canadians	NS	[152]
	T2DM	Hispanic Americans	NS	[153]
	T2DM	French Caucasian	NS	[154]
	T2DM	Korean	NS	[155]
	T2DM	Caucasians	S	[154]
	T2DM	Spanish	NS	[156]
	IGT	European/ Canadian	NS	[157]
	Non-T2DM	Japanese	NS	[158]
	Obesity	Swedish	NS	[159]
	T2DM	Caucasian Italians	NS	[160]
	T2DM	Caucasian Italians	NS	[161]
	T2DM	Pima Indians	NS	[162]
	T2DM	European Caucasians	NS	[163]
	T2DM	French Caucasians	S	[164]
+10211 T/G	T2DM	Asian Indians	S	[165]

S: Significant; NS: Non-significant; T2DM: Type 2 diabetes; SNPs: Single nucleotide polymorphisms; MS: Metabolic syndrome; IGT: Impaired glucose tolerance.

lationship to body mass index, WHR, food habits and family history. Different correlation with lipid profile and response to anti-diabetic drugs are additional indications of a genetic predisposition. SNPs in specific genes which show considerable levels of variation amongst ethnic groups around the world have been implicated in the pathogenesis of diabetes. Therefore, identification of polymorphic variants of cytokine genes in different populations and the genotypic associations between SNPs and gene-gene interactions will have clinical importance as indicators of T2DM susceptibility. Association studies of cytokine genes will help in the development of prognostic markers to identify individuals at risk. The prognostic regimens arising from such genetic studies will alter and ease out treatment strategies for T2DM and related complications. Individuals at risk will be able to take prior precautionary measures and avoid or delay the onset of the disease.

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P- Reviewer: Barzilay JI, Balamuthusamy S, Hegardt FG, Trachtman H, Tsilibary PEC
S- Editor: Wen LL **L- Editor**: Roemmele A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Novel treatment approaches in hypertensive type 2 diabetic patients

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Received: March 14, 2014 Revised: May 6, 2014
Accepted: June 10, 2014
Published online: August 15, 2014

Abstract

Type 2 diabetes mellitus (T2DM) and hypertension represent two common conditions worldwide. Their frequent association with cardiovascular diseases makes management of hypertensive patients with T2DM an important clinical priority. Carvedilol and renal denervation are two promising choices to reduce plasma glucose levels and blood pressure in hypertensive patients with T2DM to reduce future complications and improve clinical outcomes and prognosis. Pathophysiological mechanisms of both options are under investigation, but one of the most accepted is an attenuation in sympathetic nervous system activity which lowers blood pressure and improves insulin sensitivity. Choice of these therapeutic approaches should be individualized based on specific characteristics of each patient. Further investigations are needed to determine when to consider their use in clinical practice.

Key words: Diabetes mellitus; Carvedilol; Renal denervation; Insulin resistance; Glucose; Hypertension; Metabolic disorders; Ablation

Core tip: Type 2 diabetes mellitus and hypertension are two common conditions worldwide which increase the risk of cardiovascular disease with resulting disabilities and mortality. Carvedilol and renal denervation are two promising therapies to decrease insulin resistance and lower blood pressure by attenuating sympathetic nervous system activity. This review examines the clinical reports of these novel approaches.

Original sources: Castro Torres Y, Katholi RE. Novel treatment approaches in hypertensive type 2 diabetic patients. *World J Diabetes* 2014; 5(4): 536-545 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/536.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.536

INTRODUCTION

Type 2 diabetes mellitus (T2DM) and hypertension (HTN) represent two common conditions worldwide. They increase the risk for the development of cardiovascular diseases with adverse clinical outcomes including disabilities and mortality[1]. The International Diabetes Federation reports that diabetes kills one person every six seconds and afflicts 382 million people worldwide. The federation estimates that the number of people affected by the disease is expected to climb to 592 million by 2035[2].

DM is a group of metabolic diseases characterized by impairment in glucose, lipid and protein metabolism, resulting from alterations in insulin secretion, insulin action or both. While four types of DM have been classified, T2DM is the most prevalent and accounts for 90% to 95% of all diagnosed cases[3-6]. Its pathophysiology includes an increase in insulin resistance (IR) in tissues with subsequent relative insulin deficiency[7]. A great number of T2DM patients suffer from associated car-

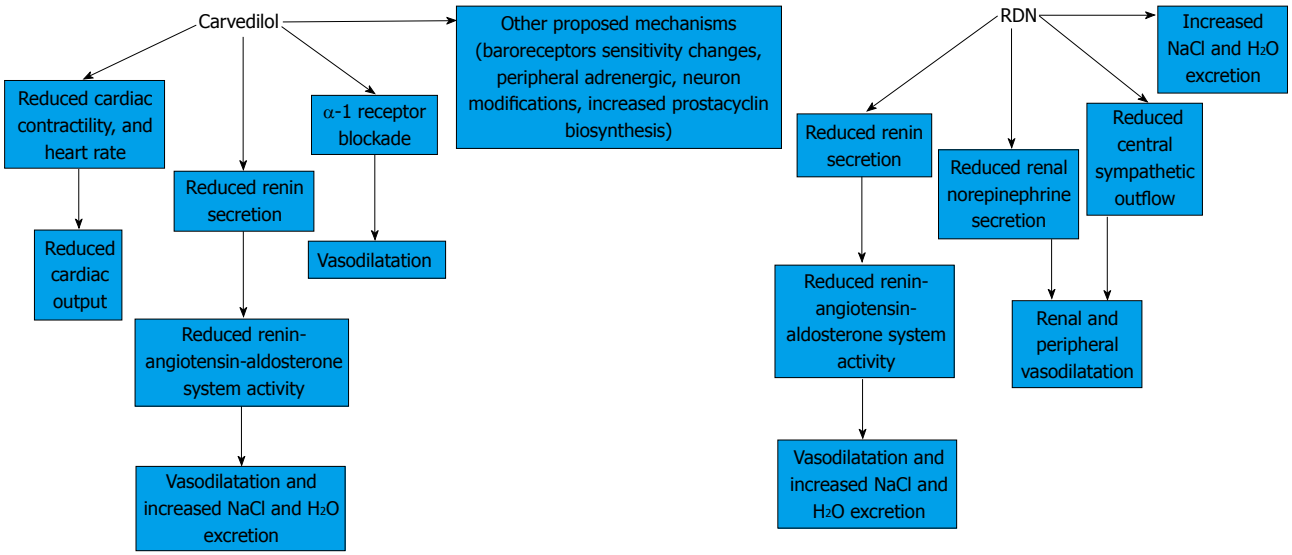

Figure 1 Antihypertensive mechanisms of carvedilol and renal denervation.

diovascular diseases. One of the most common is HTN. Over 60% of patients with T2DM have HTN[8] with resulting four-fold increased cardiovascular risk and death from complications[9,10].

Initial recommended treatment of HTN in patients with T2DM is angiotensin- converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). In the absence of cardiac comorbidity, traditional beta-blockers which increase IR do not constitute an initial choice for the treatment of HTN in patients with T2DM[4]. However, carvedilol which is a third-generation beta-blocker in some studies has demonstrated efficacy to reduce plasma glucose levels and IR[11-13] in patients with and without T2DM. Also in recent investigations, renal denervation (RDN) by catheter using radiofrequency energy has been associated with a decrease in IR in T2DM patients with an improvement in glucose control[14,15]. With both therapies the fall of plasma glucose concentrations and a reduction in blood pressure is likely due to an attenuation in sympathetic nervous system activity. Figure 1 reviews proposed antihypertensive mechanisms of carvedilol and RDN. These observations could open new choices to manage hypertensive T2DM patients with the use of one or both treatments. The benefit of improving patients' blood pressure would be complemented with an IR reduction, decreasing significantly the risk of future complications.

In this article we will review studies which suggest that carvedilol and RDN improve glucose metabolism as well as lower blood pressure in hypertensive patients with T2DM.

STUDIES THAT OBSERVED THAT CARVEDILOL IMPROVED GLUCOSE CONTROL IN HYPERTENSIVE PATIENTS WITH T2DM

It is well recognized that traditional beta-blockers have negative effects on glucose and IR[16]. In contrast, studies have demonstrated that carvedilol stabilizes plasma glucose levels and decreases IR, suggesting a novel therapeutic option in hypertensive patients with T2DM.

Carvedilol is a third-generation, nonselective beta-blocker that also possesses alpha-1 adrenergic blocking, antioxidant and calcium antagonist properties. It is a racemic lipophilic aryloxypropanolamine that causes both precapillary vasodilatation and is devoid of intrinsic sympathomimetic activity[17-20]. Carvedilol is absorbed rapidly after oral administration and it is cleared by aromatic-ring oxidation and glucuronidation in the liver. Compared with traditional beta-blockers, carvedilol has the same pharmacological actions of reducing heart rate and blood pressure[21-23]. Due to these properties, carvedilol has been used in the treatment of heart failure[24,25], angina pectoris[26,27], to improve cardiac function after myocardial infarction[28] and to reduce infarct size following myocardial ischemia and reperfusion injury[29]. Carvedilol is indicated for treating patients with congestive heart failure and after myocardial infarction with ejection fractions less than 40 percent because it has been shown to decrease mortality.

In general, traditional beta-blockers in hypertensive trials have been found to increase IR, facilitate weight gain and raise triglyceride levels. The metabolic benefits of carvedilol administration on plasma glucose reduction in patients with and without DM have been studied over many years and the results are summarized in Table 1 and discussed below.

Ehmer *et al*[30] conducted a study in non-insulin-dependent patients with DM with the aim to compare the antihypertensive effects and the influence on carbohydrate metabolism of carvedilol *vs* metoprolol tartrate. The results after eight weeks showed similar blood pressure reduction and in both groups plasma glucose concentrations remained within normal limits and glycated hemoglobin was unchanged.

Giugliano *et al*[12] compared the metabolic and cardio-

Table 1 Studies which observed glucose reduction carvedilol

Ref.	Study design	Participants	Main results
Ehmer *et al*[30]	Prospective randomized open parallel group trial	49 non-insulin-dependent diabetics with mild to moderate HTN (carvedilol *n* = 25, metoprolol *n* = 24)	Blood glucose concentrations were maintained within narrow limits. Glycated haemoglobin A1 remained unchanged. There was a reduction in blood pressure in both groups
Giugliano *et al*[12]	Prospective single-blind randomized trial	45 patients with non-insulin-dependent DM and HTN (carvedilol *n* = 23, atenolol *n* = 22)	Patients treated with carvedilol had improved glucose and lipid metabolism and reduced lipid perioxidation compared to atenolol. Both reduced blood pressure
Bakris *et al*[11]	Prospective double-blind randomized trial	GEMINI study, 1235 patients with HTN and T2DM (carvedilol *n* = 498, metoprolol tartrate *n* = 737)	The mean glycosylated hemoglobin increased with metoprolol, but not with carvedilol. An improvement of insulin sensitivity was seen with carvedilol but not with metoprolol
Phillips *et al*[32]	Prospective double-blind randomized trial	GEMINI study 1235 patients with HTN and T2DM (carvedilol *n* = 498, metoprolol tartrate *n* = 737)	After and adjustment for age carvedilol was superior than metoprolol reducing baseline glycosylated hemoglobin and also in female patients. In black people carvedilol showed a reduction in IR greater than metoprolol
Kveiborg *et al*[40]	Prospective randomized open parallel group trial	19 patients with T2DM (metoprolol succinate *n* = 10, carvedilol *n* = 9) and 10 controls	Treatment with carvedilol did not change insulin-stimulated endothelial function, whereas it deteriorated with metoprolol
Torp-Pedersen *et al*[46]	Prospective double-blind randomized trial	3029 patients with chronic heart failure and T2DM (carvedilol *n* = 1511, metoprolol tartrate *n* = 1518)	Fewer patients treated with carvedilol developed T2DM than with metoprolol
Wai *et al*[47]	Observational cohort trial	125 patients with T2DM and heart failure (carvedilol *n* = 80, bisoprolol *n* = 45)	Carvedilol significantly improved glycemic control in subjects with heart failure and T2DM
Basat *et al*[48]	Prospective double-blind randomized trial	59 patients with ST-elevation myocardial infarction (carvedilol *n* = 26, metoprolol *n* = 31)	After myocardial infarction, carvedilol added to background therapy improved insulin resistance and lipid profile

T2DM: Type 2 diabetes mellitus; HTN: Hypertension.

vascular effects of carvedilol *vs* atenolol in non-insulin-dependent T2DM hypertensive patients. Reduction in blood pressure was similar with carvedilol and atenolol, but the patients that received treatment with carvedilol had better metabolic responses. Over 24 wk, fasting plasma glucose, insulin and triglycerides levels decreased with carvedilol and increased with atenolol. In addition, an increase in high-density lipoprotein cholesterol level and decrease in lipid peroxidation was seen with carvedilol but not seen with atenolol. By improving glucose and lipid metabolism and reducing lipid peroxidation, the authors suggested that carvedilol may offer advantages in hypertensive patients with T2DM. The benefits of lipid reduction in high cardiovascular risk patients with DM have been demonstrated. In patients with DM the use of simvastatin resulted in a reduction in total mortality (43%), major coronary heart disease events (55%) and all atherosclerotic events (37%) and these reductions were greater than in non-diabetic patients[31]. In most guidelines, traditional beta-blockers are not recommended in hypertensive T2DM patients due to impairment in metabolic control and worsening lipid profile[4]. In contrast, carvedilol lowers blood pressure, improves glucose control and lipid profile, and, thus, is a unique choice in treating hypertensive T2DM patients.

An advance in this field was when researchers published the results of the GEMINI Trial which compared the glycemic and metabolic effects of carvedilol *vs* metoprolol tartrate in patients with HTN and T2DM already receiving renin-angiotensin system blockade[11]. This was a randomized, double-blind study, carried out in 1235 participants. Patients were randomized to receive a 6.25 to 25 mg dose of carvedilol (n = 498) or 50 to 200 mg dose of metoprolol tartrate (n = 737), each twice daily in addition to renin-angiotensin system blockers to achieve blood pressure goal of 130/80 mmHg. After a follow up of 35 wk, the mean of glycosylated hemoglobin increased with metoprolol [0.15% (0.04%); $P < 0.001$] but not with carvedilol [0.02% (0.04%); $P = 0.65$]. Also an improvement of insulin sensitivity was seen with carvedilol (-9.1%; $P = 0.004$) but not with metoprolol tartrate (-2.0%; $P = 0.48$). This study supports the previous benefits observed with the use of carvedilol to improve glucose control in hypertensive patients with T2DM. Particularly in this work, carvedilol associated with simultaneous administration of renin-angiotensin system blockers was superior to metoprolol tartrate to achieve this objective. In patients with diabetes, traditional beta-blockers have been shown to increase fasting glucose, increase hemoglobin A1C, facilitate weight gain and increase triglycerides by approximately thirteen per cent. In the GEMINI Trial, hypertensive diabetic patients receiving renin-angiotensin system blockade and receiving carvedilol demonstrated stabilization of glycemic control, improvement of IR, less effect on triglycerides and less development of microalbuminuria. This study supports earlier investigations suggesting that carvedilol is uniquely different than traditional beta-blockers.

More recently an extension of the GEMINI investigation was published analyzing treatment differences in subgroups on glycemic control comparing carvedilol and metoprolol tartrate in diabetic hypertensive patients

on renin-angiotensin system blockers[32]. Data analyses revealed that both carvedilol and metoprolol patients had significant and similar reductions in blood pressure. After adjustment for age there was a significant treatment benefit favoring carvedilol over metoprolol from change in baseline in glycosylated hemoglobin (0.022% *vs* 0.057%, $P = 0.003$) and IR (-9.09% *vs* -1.76%, $P = 0.015$). Female patients who received carvedilol were favored with a reduction in baseline glycosylated hemoglobin (-0.04% *vs* 0.16%, $P = 0.003$). In regard to race, carvedilol showed better results than metoprolol in African Americans patients from baseline in HOMA IR levels (-17.0% *vs* 8.2%, $P = 0.01$). The fact that carvedilol showed good blood pressure reduction and reduced glycosylated hemoglobin and IR in African American patients has important clinical implications. African Americans represent a special hypertensive group with a poor prognosis and with increased risk to develop additional complications, which are associated with the existence of frequent comorbidities and genetic predispositions[33-36]. African American T2DM hypertensive patients frequently have poor blood pressure responses to renin-angiotensin system blockers[37-39]. The GEMINI results suggest that carvedilol may be useful in the treatment of hypertensive African American patients with T2DM. Carvedilol has the potential of achieving better metabolic control, reducing blood pressure with few side effects, and improve clinical outcomes. This option needs further investigation, but this study should stimulate future work in these patients.

In further support for the unique properties of carvedilol, Kveiborg *et al*[40] examined the effects of carvedilol and metoprolol tartrate on insulin-stimulated endothelial function in patients with T2DM. These results also support the benefit of carvedilol compared with metoprolol observed in earlier studies. Treatment with carvedilol did not change insulin-stimulated endothelial function, whereas it deteriorated with metoprolol. IR is recognized as a pathophysiological cause of glucose disorders in patients with T2DM[7] and there are many reports about the relationship between this metabolic disorder and cardiovascular diseases[41,42]. Since traditional beta-blockers confer negative metabolic effects, carvedilol should be considered in the long term treatment of patients with cardiovascular disease[43-45].

Carvedilol also has been examined in the development of new onset of T2DM in patients with congestive heart failure. A total of 3029 patients with chronic heart failure were randomly assigned treatment with carvedilol or metoprolol tartrate. Fewer patients who received carvedilol were diagnosed with T2DM (119/1151 or 10.3%), compared to the metoprolol group (145/1147 or 12.6%) (HR = 0.78, CI: 0.61 to 0.997; $P = 0.048$)[46]. The results suggest that T2DM and other metabolic disorders could be avoided or at least delayed with administration of carvedilol in patients at risk.

Another study evaluated the use of carvedilol in patients with systolic heart failure[47]. Carvedilol did not affect glycemic control in patients with T2DM and additionally it had a neutral effect on lipid profile and albuminuria status, confirming earlier observations.

Basat *et al*[48] studied 59 patients after a myocardial infarction to compare the effects of carvedilol *vs* metoprolol tartrate on IR and serum lipid. After 12 wk of treatment, carvedilol showed a significantly greater reduction in insulin, C-peptide, total cholesterol and triglyceride levels than metoprolol. The authors concluded than carvedilol could constitute an option to improve IR and lipid profile in patients after myocardial infarction. In patients with coronary artery disease and specifically in those after myocardial infarction, both poor glycemic control and lipid profile are well-known risk factors which increase the number of complications and impair the prognosis[49,50]. Choosing carvedilol in these high risk patients appears indicated because of its unique metabolic advantages compared to traditional beta- blockers.

STUDIES THAT OBSERVED THAT RDN IMPROVED GLUCOSE CONTROL IN HYPERTENSIVE PATIENTS WITH T2DM

RDN has emerged as a promising treatment for HTN[51-55]. Symplicity HTN-1[56] and HTN-2[57] studies demonstrated the efficacy and safety of RDN in patients with resistant HTN. State-transition modeling suggests that RDN is a cost-effective strategy for resistant HTN that can reduce the risk of stroke, myocardial infarction, coronary heart disease, heart failure and end-stage renal disease[58]. Another study suggests that potential lifetime cost-effectiveness ratios may be increased when RDN is performed earlier in patients with resistant HTN[59]. Follow-up of Symplicity patients demonstrate a durable blood pressure reduction out to 36 mo[60].

The principles of catheter-based RDN are based on the influence of afferent and efferent renal nerves in blood pressure physiopathology. As shown in Figure 1, after an ablation of renal nerves there is a reduction in blood pressure, sympathetic nervous system activity and renin-angiotensin system activity and increase in water and salt excretion[61].

Based on these observations, some investigators have examined catheter-based RDN on glucose control. Table 2 describes studies which observed glucose reduction after RDN. These studies were based on the knowledge that sympathetic overactivity can induce IR and hyperinsulinemia. Mahfoud *et al*[14] designed an investigation which enrolled 50 patients with resistant HTN. The group study ($n = 37$) received bilateral catheter-based RDN and the control group ($n = 13$) was assigned to continue medical therapy. Three months after treatment fasting glucose was reduced in the RDN group from 118 ± 3.4 to 108 ± 3.8 mg/dL ($P = 0.039$). Insulin levels were decreased from 20.8 ± 3.0 to 9.3 ± 2.5 μIU/mL ($P = 0.006$) and IR decreased from 6.0 ± 0.9 to 2.4 ± 0.8 ($P = 0.001$). Mean 2-h glucose levels during oral glucose tolerance testing were also reduced significantly by 27 mg/dL

Table 2 Studies which observed glucose reduction after renal denervation

Ref.	Study design	Participants	Main results
Mahfoud *et al*[14]	Prospective, controlled unblinded, randomized study	50 patients with resistant HTN (37 patients underwent catheter-based RDN and 13 patients in a control group	RDN improved glucose metabolism and insulin sensitivity in addition to a significantly reducing blood pressure
Witkowski *et al*[65]	Prospective, nonrandomized, open-label study	10 patients with refractory hypertension and sleep apnea (7 men and 3 women, who underwent RDN)	RDN reduced blood pressure and improved glucose metabolism

HTN: Hypertension: RDN: Renal denervation.

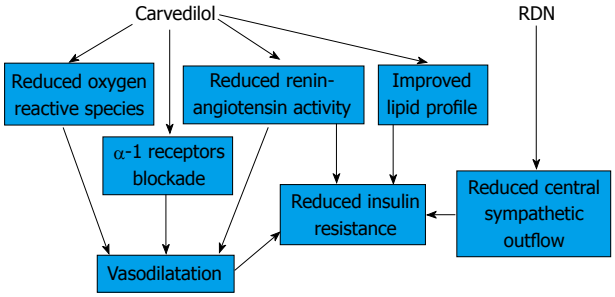

Figure 2 Proposed mechanisms to explain decreased insulin resistance with carvedilol and renal denervation in type 2 diabetes mellitus patients with hypertension.

(P = 0.012) while there were no significant changes in BP or any of the metabolic markers in the control group. These excellent results in metabolic control were accompanied by a significant reduction in blood pressure. This was the first study proving the efficiency of RDN to reduce IR and improve glycemic control. RDN represents one of the most promising non pharmacological strategies to treat HTN, thus, the possibility observed in this research to reduce blood pressure and concomitant IR may open new options for patients.

Guidelines of some societies recommend that patients who receive RDN continue antihypertensive medical therapy after the procedure because the blood pressure often decreases slowly[62,63]. In this study it is suggested that the improvements seen in glucose control are due to a reduction in central sympathetic outflow after RDN. If further studies support this concept in patients with T2DM other conditions with IR like obesity merit study[64].

There is further support for the concept than RDN may benefit glucose control. Other investigators have examined the effects of RDN on blood pressure, sleep apnea course, and glycemic control in patients with resistant HTN and sleep apnea. RDN decreased blood pressure, attenuated sleep apnea severity and decreased two hour post prandial plasma glucose and glycosylated hemoglobin levels[65].

PROPOSED MECHANISMS TO EXPLAIN A PLASMA GLUCOSE REDUCTION FROM CARVEDILOL AND RDN

There are several mechanisms as shown in Figure 2 that may explain improved glycemic control with the use of carvedilol and RDN.

Traditional beta-blockers cause an increase in peripheral vascular resistance due to unopposed alpha vasoconstriction with resultant reduced glucose disposal to skeletal muscles and reduction in glucose uptake[66]. Carvedilol has alpha-1 blocker properties that causes vasodilatation and maintenance of blood flow to skeletal muscles. This difference may explain in part carvedilol's actions on glucose control compared to traditional beta-blockers.

Another mechanism by which carvedilol may improve glucose control is by reducing oxygen reactive species. T2DM is associated with endothelial dysfunction with increased reactive oxygen species and decreased endothelial nitric oxide synthase activity[67]. This phenomenon causes a reduction in oxide nitric availability with resultant vasoconstriction. Giugliano *et al*[12] found an increase in insulin sensitivity with a concomitant reduction in oxidative stress in patients with T2DM treated with carvedilol. Because carvedilol has antioxidant properties it appears to decrease reactive oxygen species and improve endothelial function. Other investigators have also found that carvedilol significantly reduced oxidative stress and C-reactive protein levels in hypertensive patients[68] and increased activity of antioxidant enzymes in diabetic rats[69].

On the other hand there are studies which have demonstrated that IR is related to an increase in sympathetic nervous system activation. An increase in sympathetic nerve activity and HTN in Caucasians with IR has been observed[70]. T2DM and HTN are known to be closely linked with increased sympathetic nervous activity and IR[71,72]. Reflex sympathetic activation has been shown to induce acute IR in the human forearm[73]. Carvedilol causes a significant reduction in cardiac and systemic norepinephrine spillover and this effect was not seen with other beta-blockers like metoprolol[74,75]. The relationship between an increase in sympathetic nervous activity and the development of IR, and the ability of carvedilol to reduce systemic norepinephrine may in part explain the findings of this drug reducing glucose levels. Similar results reducing norepinephrine spillover have been seen with the use of catheter-based RDN[56]. Increased sympathetic nervous system activity in tissues can result in IR. There is evidence of impaired ability of the cells to transport glucose through their membranes due to a decrease in blood flow after a rise in noradrenaline concentration[73]. The mechanism could be related to an

Table 3 Comparison between carvedilol and renal denervation as therapeutic choices to reduce blood pressure and glucose levels in hypertensive type 2 diabetes mellitus patients

Therapeutic method	Mechanism of action	Medical indication	Mechanisms which explain glucose reduction	Contraindications	Side effects
Carvedilol	α1, non-selective β-blocker, antioxidant and calcium antagonist properties[17-20]	Treatment of hypertension[21] heart failure[25] and coronary artery disease[27]	An improvement in insulin sensitivity by a reduction in sympathetic nerve activity[74,75] and free radicals[68,69]	Bronchial asthma, second-third degree atrioventricular block, sick sinus syndrome, severe bradycardia, patients with severe cardiogenic shock and heart failure who use inotropic drugs and hepatic impairement[17-20]	Frequent: edema, dizziness, bradycardia, hypotension, nausea, diarrhea and blurred vision Rare: deterioration of renal and hepatic function[17-20]
RDN	Ablation of afferent and efferent renal nerves[51-55]	Treatment of resistant hypertension[56,57]	An improvement in insulin sensitivity by reduction in sympathetic nerve activity[56,57]	Polar or accessory arteries, renal artery stenosis, prior renal revascularization and glomerular filtration rate < 45 mL/min per 1.73 m^2[56,57,62]	Renal artery dissection, postprocedural hypotension, femoral artery pseudoaneuryn, intraprocedural bradycardia[56,57]

increased distance that insulin has to travel from intravascular compartment to cell membranes due to a reduction of number of open capillaries as a consequence of vasoconstriction by sympathetic overactivity.

Another mechanism by which carvedilol may improve glucose control could be through the positive effects of carvedilol improving lipid profile. There appears to be a direct relationship between free fatty acids and IR. It is not fully understood why high plasma levels of fatty acids can produce IR, but a proposed mechanism is that permanent increases in plasma free fatty acids results in an intracellular accumulation of triglycerides and other compounds involved in triglyceride synthesis. Some of these compounds can activate a novel protein kinase C, and this protein is able to cause IR by decreasing tyrosine phosphorylation of the insulin receptor substrates[76-78]. Thus, the improvement in lipid profile observed with carvedilol[11,12] may in part explain, its ability to increase insulin sensitivity and subsequently improve glucose control.

Both carvedilol and RDN appear to reduce glucose levels by a decrease in IR and this change is associated with a reduction in sympathetic nervous system activity. However, beyond this possible relationship there are other possible mechanisms to explain improved glucose control after administration of carvedilol. Further investigations are needed to understand the metabolic pathways resulting in improved glucose control with the use of carvedilol and RDN.

COMPARISON BETWEEN CARVEDILOL AND RDN TO REDUCE GLUCOSE LEVELS

A comparison between carvedilol and RDN as options to reduce blood pressure and glucose levels in T2DM hypertensive patients is listed in Table 3. While carvedilol is administrated as an oral medication which requires patient's adherence, RDN is an interventional procedure whose safety and durability is still under investigation. Clinical trial data from Symplicity radiofrequency catheter systems have created much interest in the role of the renal nerves in HTN and other conditions such as diabetes mellitus. Furthermore, the attenuation of blood pressure observed has led to the rapid development of alternative methods of RDN by radiofrequency ablation as well as by ultrasound ablation and peri-vascular pharmacologic ablation. Many trials investigating these various innovative approaches to achieve RDN are ongoing. The factors which should be examined when considering carvedilol and/or RDN are the efficacy, safety and cost. Also, physicians need to individualize the recommended treatment because depending on physiological characteristics patient responses (and benefits) will vary.

PERSPECTIVE

Patients with HTN and T2DM require long term therapy. Thus, choice of antihypertensive agents results in long term risks and benefits. Initial recommended treatment of HTN in patients with T2DM is ACE inhibitors or ARBs which have favorable effects on carbohydrate metabolism and insulin resistance. Long-acting dihydropyridines have a neutral effect on glucose metabolism and insulin resistance. In contrast, thiazide-type diuretics can cause hyperglycemia and traditional beta-blockers can increase IR. Furthermore, hypertensive patients with increased cardiovascular risk may require 3-hydroxy-methylglutaryl coenzyme A reductase inhibitors, or statins, which appear (with the exception of pravastatin) to increase the risk of patients developing T2DM. Carvedilol and RDN appear to improve insulin sensitivity and glucose metabolism as well as lower blood pressure. Some guidelines recognize carvedilol's unique metabolic advantages compared to traditional beta-blockers and recommend its use in patients with HTN and T2DM if blood pressure goals have not been achieved using ACE inhibitors or ARBs. Carvedilol has been shown to stabilize HbA1c, improve insulin resistance, and slow development of microalbuminuria in the presence of renin-angiotensin system blockade compared with metoprolol tartrate[11].

Use of carvedilol should be individualized in patients with HTN and T2DM. In general, beta-blockers may mask some of the manifestations of hypoglycemia, particularly tachycardia. Nonselective beta-blockers may potentiate insulin-induced hypoglycemia and delay recovery of serum glucose levels. Patients subject to spontaneous hypoglycemia, or diabetic patients receiving insulin or oral hypoglycemic agents, should be cautioned about these possibilities. Furthermore, beta-blockers can precipitate or aggravate symptoms of arterial insufficiency in patients with peripheral vascular disease. Caution should be exercised in such individuals.

Presently RDN should only be considered in patients with resistant hypertension after causes of secondary hypertension have been excluded, with fairly preserved renal function and eligible renal arterial anatomy. It is not recommended to perform RDN in patients with HTN and T2DM outside of appropriately designed clinical trials.

CONCLUSION

Carvedilol and RDN improve glucose metabolism and insulin sensitivity in parallel with blood pressure reduction. These novel approaches may therefore provide benefit in patients with resistant HTN and T2DM who are at high cardiovascular risk and have not reached recommended goals to improve endothelial function and preserve renal function. An attenuation in sympathetic nervous system activity is the most likely mechanism to explain these actions. There have been no head-to-head comparisons, but RDN appears to have a greater effect on glucose metabolism than carvedilol. Further investigations and follow up are needed to determine the long-term durability of RDN, its efficacy in other diseases such as heart failure, stroke and kidney failure, and its use in stage 1 HTN. Currently, there are no clinical trial data available to indicate that RDN improves cardiovascular outcomes. If further trials confirm blood pressure lowering and improved glucose metabolism with carvedilol and RDN, these approaches represent reasonable choices for the treatment of patients with HTN and T2DM who have not reached guideline goals. These novel approaches could be used together to reach goals. Use of these novel treatments should be individualized in patients taking into account efficacy, safety, and cost.

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P- Reviewer: Hung MJ, Nakos G **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Peripheral arterial disease, type 2 diabetes and postprandial lipidaemia: Is there a link?

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Received: January 24, 2014 Revised: March 19, 2014
Accepted: July 17, 2014
Published online: October 15, 2014

Abstract

Peripheral arterial disease, manifested as intermittent claudication or critical ischaemia, or identified by an ankle/brachial index < 0.9, is present in at least one in every four patients with type 2 diabetes mellitus. Several reasons exist for peripheral arterial disease in diabetes. In addition to hyperglycaemia, smoking and hypertension, the dyslipidaemia that accompanies type 2 diabetes and is characterised by increased triglyceride levels and reduced high-density lipoprotein cholesterol concentrations also seems to contribute to this association. Recent years have witnessed an increased interest in postprandial lipidaemia, as a result of various prospective studies showing that non-fasting triglycerides predict the onset of arteriosclerotic cardiovascular disease better than fasting measurements do. Additionally, the use of certain specific postprandial particle markers, such as apolipoprotein B-48, makes it easier and more simple to approach the postprandial phenomenon. Despite this, only a few studies have evaluated the role of postprandial triglycerides in the development of peripheral arterial disease and type 2 diabetes. The purpose of this review is to examine the epidemiology and risk factors of peripheral arterial disease in type 2 diabetes, focusing on the role of postprandial triglycerides and particles.

Key words: Peripheral arterial disease; Type 2 diabetes; Postprandial lipidaemia; Apolipoprotein B-48; Ankle-brachial index; Non-fasting triglycerides

Core tip: Peripheral arterial disease is highly prevalent in type 2 diabetes; traditional risk factors contribute to the disease. Interestingly, postprandial lipidaemia is increased in both conditions. However, one study showed that only subjects with both type 2 diabetes and peripheral arterial disease had elevation of postprandial lipids; subjects with type 2 diabetes and a normal ankle-brachial index had a normal postprandial response. Because most of the triglycerides of chylomicrons are extracted in muscle and adipose cells in the legs, the authors speculate on whether arteriosclerosis in the legs may contribute to greater postprandial lipidaemia.

Original sources: Valdivielso P, Ramírez-Bollero J, Pérez-López C. Peripheral arterial disease, type 2 diabetes and postprandial lipidaemia: Is there a link? *World J Diabetes* 2014; 5(5): 577-585 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/577.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.577

EPIDEMIOLOGY OF PERIPHERAL ARTERIAL DISEASE IN TYPE 2 DIABETES MELLITUS

Peripheral arterial disease (PAD) is produced by narrowing of the calibre of the medium-sized arteries and its widest

Table 1 Prevalence of peripheral arterial disease in Spanish cohorts

Study	Number of subjects	Age (yr)	Study population		ABI < 0.9 (%)
HERMEX[17]	2833	51	General	All	3.7
				Without diabetes	2.8
				With Diabetes	6.2
ESTIME[6]	1324	68	General	All	8
				Without diabetes	6.6
				With diabetes	19
MERITO[19]	1519	66	Internal medicine outpatient clinic	SCORE ⩾ 3	26.2
				With Diabetes	26.1
VITAMIN[20]	493	68	Internal medicine outpatient clinic	Without DM2	21
				With DM2	38
ARPTER[18]	3171	63	General	All	6.4
				Without diabetes	5.4
				With diabetes	12.6
REGICOR[21]	6262	56	General	All	4.5
				Without diabetes	4
				With diabetes	8.4
FUENCARRAL Health Center[22]	1360	70	Primary health care centre	Without diabetes	4.3
				With diabetes	11.3
ALBACETE[23]	784	61	General	All	10.5
				Without diabetes	9
				With diabetes	19
RONDA PRIM Health Center[25]	289	65	Primary health centres	Diabetes	21.5
CIUDAD JARDIN Health Center[78]	456	61	Primary health centre	Diabetes	27
PADiD Study[24]	1462	78	Internal medicine outpatient clinics	Diabetes	60
MARINA BAIXA Hospital[89]	360	67	Internal medicine outpatient clinics	Diabetes	27

ABI: Ankle-brachial index.

definition encompasses all extracoronary and extracerebral vascular disease. However, the term PAD is usually restricted to involvement of the lower limbs, particularly in the iliac bifurcation, and the iliofemoral and popliteal arteries[1]. The main cause of arterial stenosis in developed countries is atherosclerosis.

The prevalence of PAD in Europe and the United States is estimated to be 27 million persons[2]. The prevalence of PAD increases progressively with age, with most cases starting after the age of 40 years. It is well known that only a very few PAD patients actually have symptoms, around 10%-20%[3]. The use of a standardized questionnaire in the physician's office can increase the detection of claudicant patients[4,5]. Most patients with PAD are identified from non-invasive tests, such as the ankle-brachial index (ABI). Using this widely extended technique in Spain led to the identification of PAD in 8% of individuals aged 55-85 years[6]. In addition to age, the other cardiovascular risk factors also increase the likelihood of developing PAD. Thus, in persons with a low cardiovascular risk the prevalence of PAD is almost inconsiderable[7], whereas it can reach 27% in persons with type 2 diabetes[8].

The prognosis for patients with PAD, both symptomatic and asymptomatic, is poor[9]. Overall mortality is increased and the risk of death is even greater than that in patients who have angina or acute myocardial infarction[10-13]. Data from Spain confirm these findings. An analysis of the FRENA, REACH and AIRVAG registries showed that patients with PAD have a greater frequency of symptomatic multivessel disease and a worse one-year prognosis than patients with single-vessel involvement or cerebrovascular disease[14].

Diabetes and PAD

Diabetes, together with smoking, is the main risk factor for PAD[15]. Of patients who attended an angiology office in Spain due to intermittent claudication and who underwent arterial surgery or had an ABI ⩽ 0.9, 67% had diabetes mellitus[16]. Population-based studies in Spain, undertaken in either the general population or at various levels of care, showed that the presence of diabetes mellitus doubled or even tripled the possibility of having PAD (Table 1)[6,17-23]. The prevalence of an ABI < 0.9 in series of Spanish patients with diabetes ranges from 21% to 60% (Table 1)[8,24,25]. In the autonomous communities of Andalusia and the Canary Islands, 72% of all lower-limb amputations between 1996 and 2006 involved patients with diabetes[23,26,27]. In patients with diabetes, for every 1% increase in haemoglobin A1c there is a corresponding 26% increased risk of PAD[28]. The presence of PAD also increases the risk of death in patients with diabetes mellitus[29,30]. The prognosis for PAD is worse in patients with diabetes than those without diabetes[31].

Diagnosis of PAD in diabetes

The diagnosis of PAD usually depends on the sum of the symptoms, particularly intermittent claudication, plus the physical examination, especially the lack of pulses and the trophic disorders leading to critical limb ischaemia and distal necrosis[32]. However, patients, particularly diabetic patients, commonly have other processes at

the same time that can alter the traditional symptoms of PAD, making them much less specific[33]. Accordingly, the measurement of the ratio of the systolic blood pressures in the ankle and the arm, the ABI, has been recommended as the screening method for asymptomatic PAD and as a form of confirmation in symptomatic PAD[2,34,35]. A finding in one limb of an ABI < 0.9 with the measurement taken at rest under standard conditions is considered diagnostic of PAD, with an ABI between 0.9 and 1.0 considered borderline[36].

One limitation of the ABI, especially relevant in patients with diabetes, is arterial media calcification, which can lead to non-compressible arteries (ABI > 1.4) or false normal values. A recent study showed that individuals with an ABI > 1.4 have a worse prognosis than those with a normal ABI and even those with an ABI < 0.9. The prevalence of diabetes in the group with an ABI > 1.4 was 58%, compared with 18% and 48% in those with a normal ABI or those with an ABI < 0.9[37]. It has long been known that the sensitivity of the ABI to correctly diagnose PAD is considerably reduced in the presence of arterial media calcification and that, clinically, this calcification is associated with the presence of peripheral neuropathy[38,39]. Accordingly, in the presence of peripheral neuropathy it is recommended to use an alternative method, such as flow wave analysis using Doppler colour ultrasound[40,41]. In our experience this limitation is not negligible. In a series of 456 patients with type 2 diabetes, 35 were found to have intermittent claudication (7.6%); only 22 of these had an ABI < 0.9. Of the other 13, 12 underwent colour Doppler ultrasound and in 3 (25%) we obtained a monophasic wave, diagnostic of PAD. Thus, a normal ABI does not rule out PAD in patients with type 2 diabetes, and these patients should therefore undergo complementary tests if they have symptoms suggestive of PAD[8].

The resting ABI should be used as the diagnostic technique for PAD when lower limb arteriosclerosis is suspected. This should be done in persons with one or more of the following: symptoms in the lower limbs after exercise, wounds with delayed healing, and individuals older than 65 years of age or older than 50 years with a history of smoking or diabetes[34]. Given the high prevalence of PAD in patients with diabetes, the ADA recommends screening with the ABI in patients with diabetes who are older than 50 years and who have another risk factor (smoking, hypertension, hyperlipidaemia, or diabetes for more than 10 years)[42].

LIPIDS, POSTPRANDIAL LIPIDAEMIA AND PAD

Fasting lipids in PAD

Lipid abnormalities in PAD have received less attention than in other areas, as for example, in coronary anomalies. Very few prospective studies have focused on the relation between triglycerides and peripheral vascular disease. The most common feature of PAD is raised levels of triglycerides and lower levels of high-density lipoprotein (HDL) cholesterol as compared with age- and sex-matched controls without vascular disease, with similar levels of cholesterol and low-density lipoprotein (LDL) cholesterol[43-47]. The frequency of a cluster of lipid abnormalities of the type of raised triglycerides and small and dense LDL and reduced HDL was 20% in persons with PAD *vs* 0% in the control group[48]. Several studies have also shown that triglyceride levels are a predictive factor for PAD[49-51], though not all[52].

Postprandial lipidaemia: Atherogenic mechanism

Unlike the carbohydrates, which normally only show transitory increases after a meal, the circulating triglycerides show a pronounced increase (postprandial lipidaemia) one hour after the intake of a fat-rich meal (around 30-60 g), and can remain high for 5-8 h after the meal. As most persons regularly consume fatty meals every 4-5 h, the usual state in humans insofar as their triglyceride metabolism is concerned is clearly a continuous postprandial lipidaemic state[53,54].

The large triglyceride-transporting particles, the chylomicrons and the very low-density lipoprotein (VLDL), are too large to cross the endothelium and they therefore don't contribute to the atherosclerosis, but the same does not occur with the chylomicron remnants and the intermediate-density lipoprotein (IDL), which are much smaller particles[55]. Evidence exists that the cholesterol in the postprandial particles, originating in the intestine, contribute to the phenomenon of atherosclerosis, both in animals and in humans[56-59].

Postprandial lipidaemia and cardiovascular disease: Case-control vs prospective studies

Since the seminal work of Zilversmit, many case-control studies have found an association between the magnitude of the postprandial lipidaemia and the presence and severity of coronary artery disease[60,61]; these studies have been reviewed by Lopez-Miranda *et al*[62]. Prospective studies, however, are few and controversial. Reyes-Soffer *et al*[63] followed 69 patients with type 2 diabetes who were free of coronary disease for a mean of 8.7 years; 33 patients remained disease-free. No differences were found in the postprandial parameters at the initial visit between the groups, and the authors concluded that the postprandial triglycerides do not predict the onset of coronary disease in individuals with diabetes. A more recent study involving 514 survivors of an acute coronary syndrome found that the postprandial triglycerides after the oral intake of 75 g of fat predicted the appearance of new events at 18 mo. In the subgroup of patients without diabetes or oral glucose intolerance the relative increase in postprandial triglycerides was an independent predictor of events[64].

Non-fasting triglycerides

Interest in studying postprandial lipidaemia has increased over recent years as a result of studies showing that serum triglyceride levels measured in a non-fasting state have

proved to be better predictors for the risk of vascular disease than fasting triglyceride concentrations, *i.e.*, when they are quantified after 8-10 h of fasting[65-68]. Two meta-analyses also support the association between fasting and postprandial triglycerides and the vascular risk[69,70]. One of the problems encountered when introducing postprandial triglyceride measurements in the clinical setting is the absence of specific recommendations in the clinical practice guidelines and thus the identification of a threshold level above which postprandial hypertriglyceridaemia is recognised. To date, only the American Association of Clinical Endocrinologists has considered the possibility of evaluating the non-fasting triglyceride concentration[71]. Based on evidence from the above mentioned population-based studies, an expert group estimated non-fasting triglyceride levels < 180 mg/dL as desirable[72]. This means that 38% of the men and 20% of the women in the Copenhagen study who had figures above these levels have postprandial hypertriglyceridaemia[73].

Suggestion for the measurement of postprandial lipidaemia

The study of postprandial (hyper)lipidaemia has several inconveniences. The most important at present is the poor clinical yield and the great complexity of the fat test; its prolonged time is uncomfortable for both the patient and the medical personnel, not to mention the lack of standardization for the test. A few years ago, using data from a meta-analysis of 113 studies in healthy subjects by Mihas *et al*[74], an expert group attempted to standardize the test and recommended a fat tolerance test meal consisting of 75 g fat, 25 g carbohydrates and 10 g protein. Furthermore, the fatty test meal should contain mixtures of saturated and unsaturated fatty acids in a digestible form and be easy to prepare. The candidates for the test should have fasting triglycerides of 90-180 mg/dL and the test can be shortened with the measurement of the serum triglycerides at 4 h, with no need to reach a complete postprandial curve of 8 or 12 h[72].

POSTPRANDIAL LIPIDAEMIA AND PAD IN TYPE 2 DIABETES

Little attention has been given to the study of postprandial lipidaemia in patients with PAD. Only the elegant paper by Lupattelli *et al*[75] showed that the magnitude of postprandial lipidaemia, expressed as "the area under the incremental curve for triglycerides," was higher in 16 non-diabetic normolipidaemic claudicant patients with PAD than in 10 normolipidaemic control subjects, suggesting the relevance of postprandial lipoprotein metabolism in the pathogenesis of peripheral atherosclerosis. However, although normolipidaemic, the patients in Lupattelli's study had slightly higher fasting triglycerides than their controls.

In recent years our group has studied the relation between lipids and postprandial particles, PAD and type 2 diabetes mellitus. Firstly, the postprandial triglycerides were more strongly associated with PAD in individuals with type 2 diabetes mellitus than were the fasting triglycerides. A group of 119 patients with type 2 diabetes mellitus treated with just diet and/or oral glucose lowering agents, with no lipid-lowering treatment, were analyzed at fasting and 4 h after a mixed breakfast containing 50 g of fat and 40 g of carbohydrates. Although the patients with cardiovascular disease, most of them with asymptomatic PAD and identified by an ABI < 0.9, had lower fasting HDL cholesterol levels and higher triglyceride levels, only the triglycerides at 4 h post-breakfast were associated in the multivariate analysis with cardiovascular disease, together with the duration of the disease and smoking[76].

The postprandial triglycerides include not only those contained in chylomicron particles and their remnants, but also those contained in VLDL and IDL. In an attempt to further understand the role of postprandial fat in PAD, we undertook a second experiment to analyze the serum concentration of apolipoprotein B48, a protein that is only associated with chylomicrons and their remnants and is not interchanged with any other circulating particle. This second study involved 101 patients with type 2 diabetes mellitus and 73 controls without diabetes, both groups with no known cardiovascular disease. Asymptomatic vascular disease was identified from the ABI and as a marker of postprandial particles we used the apolipoprotein B48, measured with a commercial enzyme-linked immunosorbent assay. Of the patients with type 2 diabetes mellitus, 21 had PAD as defined by an ABI < 0.9, though no control had PAD. The levels of triglycerides and apolipoprotein B48, both fasting and postprandial, were significantly higher in the group of diabetic patients with PAD than in those without PAD and the controls. Curiously, no differences were found between the controls and the patients with type 2 diabetes mellitus without PAD. Of all the lipid and non-lipid parameters studied, only apolipoprotein B48 and smoking were associated with the presence of PAD in a binary logistic regression analysis. Likewise, the presence of PAD was an independent predictor of the levels of apolipoprotein B48, both fasting and 4 h after a mixed breakfast[77].

As the patients with type 2 diabetes mellitus in the previous studies did not receive any insulin or lipid-lowering therapy, we decided to confirm the findings in a larger population with type 2 diabetes mellitus without these exclusion criteria. Again, using an ABI < 0.9 as a marker of PAD, we found in 456 patients with type 2 diabetes mellitus that fasting apolipoprotein B48 was a marker of PAD, independently of the other lipid factors, statin treatment or insulin therapy[78]. Identical results have also been reported by another group[79].

May PAD delay postprandial lipid catabolism?

Taken together, these studies confirm an association between postprandial particles, measured as triglycerides 4 h after breakfast or as fasting and postprandial apolipoprotein B48, and PAD. In the above-mentioned studies, a diabetic status in itself was not associated with a greater

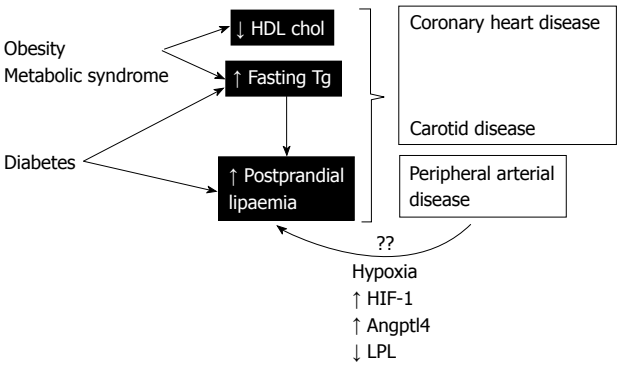

Figure 1 Proposed mechanism linking peripheral arterial disease and worsening postprandial lipaemia. HIF-1: Hypoxia-induced factor 1; Angptl4: Angiopoietin-like protein 4; LPL: Lipoprotein Lipase; HDL: high density lipoproteins.

concentration of postprandial triglycerides or apolipoprotein B48 if there was no PAD. As mentioned earlier, the case-control studies show an association between postprandial lipidaemia and cardiovascular disease, particularly coronary disease.

An explication for this association was provided by Lupattelli *et al*[75]. Somehow, and following the hypothesis of Zilversmit[80], the exposure of the endothelium to greater concentrations of postprandial particles favours the appearance of arteriosclerotic lesions, in our case in the lower limbs. Though this hypothesis is the most plausible, no causality can be deduced from the association studies. Accordingly, it is worth speculating about whether arteriosclerotic disease in the legs could alter chylomicron metabolism, slowing it. With this in mind, consideration should be given to the study by Horton *et al*[81], who showed that men have higher triglyceride concentrations than women because women posses a greater extractive capacity of triglycerides in adipose and muscle tissues in the lower limbs when they undergo a fatty breakfast. For some reason the catabolism of the chylomicrons in the legs is not negligible and an alteration in the circulation in the legs may worsen or slow this metabolism.

The kinetics of lipoproteins are marked by (1) their intestinal production; (2) hydrolysis of their triglycerides by the action of lipoprotein-lipase anchored in the endothelium (but synthesised in adipose and muscle tissue cells); and (3) removal of chylomicron remnants by hepatic receptors. These steps are all modulated by the levels and genetic variants of the apolipoproteins like C-Ⅱ, C-Ⅲ, E, A-5[82,83]. As persons with arteriosclerosis, particularly those with PAD, have a marked endothelial dysfunction[84], it is possible to speculate that the action of an enzyme anchored to the endothelium, as is the case of lipoprotein lipase (LPL), is reduced. Given the great extension of the endothelial surface in the legs (in comparison with coronary arteriosclerosis), established PAD might affect postprandial lipidaemia more intensely than coronary disease.

If this hypothesis were true, what would its mechanism of production be? The consequence of arteriosclerosis is tissue ischaemia. This is usually manifested as intermittent claudication, though the tissues may experience hypoxia in earlier stages. Tissue hypoxia leads to changes in the endothelial cells (where the LPL are anchored) or in the production of LPL (or its associated proteins) by adipose or muscle cells[85]. Cells submitted to hypoxia upregulate the expression of hypoxia-inducible factor 1, a transcription factor that induces changes in innumerable target genes that were reviewed some time ago[86]. Of note among these changes is the raised expression of angiopoietin-like 4 protein (Angptl4) and vascular endothelial growth factor (VEGF). VEGF intervenes in the processes of angiogenesis, much related with chronic ischaemia of the lower limbs and the formation of collateral vessels. Angptl4 is a potent inhibitor of LPL, the enzyme that intervenes critically in the first step of the catabolism of triglyceride-rich particles[87]. A recent experimental animal study showed that mice submitted to cyclic hypoxia experienced inhibition of the catabolism of triglyceride-rich lipoproteins as a consequence of a drastic reduction in adipose tissue LPL activity, coupled with a notable increase in Angptl4[88] (Figure 1).

Taken together, these data suggest that postprandial hyperlipidaemia, a recognised vascular risk factor associated with obesity, the metabolic syndrome and type 2 diabetes, could be aggravated by PAD, further exposing other arterial territories to greater concentrations of postprandial atherogenic particles. Finally, if the hypoxia were an underlying mechanism, it could be improved by percutaneous or surgical revascularization.

ACKNOWLEDGMENTS

Authors would like to thank to Ian Johnstone for the English edition of the manuscript.

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P- Reviewer: Barzilay JI, Neri V, Tarantino G **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Treatment of type 2 diabetes, lifestyle, GLP1 agonists and DPP4 inhibitors

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Received: January 26, 2014 Revised: July 23, 2014
Accepted: July 27, 2014
Published online: October 15, 2014

Abstract

In recent years the treatment focus for type 2 diabetes has shifted to prevention by lifestyle change and to more aggressive reduction of blood sugars during the early stage of treatment. Weight reduction is an important goal for many people with type 2 diabetes. Bariatric surgery is no longer considered a last resort treatment. Glucagon-like peptide-1 agonists given by injection are emerging as a useful treatment since they not only lower blood sugar but are associated with a modest weight reduction. The role of the oral dipeptidyl peptidase 4 inhibitors is emerging as second line treatment ahead of sulphonylureas due to a possible beneficial effect on the beta cell and weight neutrality. Drugs which inhibit glucose re-absorption in the kidney, sodium/glucose co-transport 2 inhibitors, may have a role in the treatment of diabetes. Insulin treatment still remains the cornerstone of treatment in many patients with type 2 diabetes.

Key words: Type 2 diabetes; Lifestyle modification; Dipeptidyl peptidase 4 inhibitors; Glucagon-like peptide-1 agonists; Insulin

Core tip: Treatment of diabetes is difficult. Initial success in achieving treatment goals is followed by deterioration and the necessity for additional treatments. Exciting new drugs with new modes of action, have stimulated diabetologists to strive for improved control in the knowledge that complications will be reduced or prevented. Obese patients, who loose weight on glucagon-like peptide-1 agonists are usually delighted with these drugs but for those who fail to loose weight changing to oral dipeptidyl peptidase-4 inhibitors would seem a good choice. sodium-glucose transporter-2 inhibitors have the added benefit of being effective even if blood sugar is near to target but uro-genital infection is a concern.

Original sources: Tomkin GH. Treatment of type 2 diabetes, lifestyle, GLP1 agonists and DPP4 inhibitors. *World J Diabetes* 2014; 5(5): 636-650 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/636.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.636

INTRODUCTION

Readers interested in diabetes must be sick and tired reading that diabetes is a global problem of immense size and getting worse by the day with predictions that we will all have the disease one day! I exaggerate of course but it is sad to realise that although we know so much more about the condition we have made little progress in reducing or conquering the disease. A recent history of diabetes in the past 200 years by Polonsky[1] gives an excellent review of the history of discovery of so many mechanisms that are faulty in diabetes and the number of Nobel prize winners who have contributed to such wonderful success, yet more and more people are being diagnosed with the condition/disease and the consequences are immense in terms of suffering and financial cost. One should not forget that before the discovery of insulin 90 years ago

diabetes was a rapidly fatal disease and there was little interest in what we now term type 2 diabetes. Type 2 diabetes now makes up 90% of all diabetes. Insulin resistance rather than insulin deficiency is the major player in the vast majority of type 2 diabetes and type 2 diabetes can be reversed, at least in many patients, with exercise and weight reduction. This is not new information but was highlighted by Taylor's group in Newcastle in 2011[2] when they did a very simple experiment on patients who had diabetes, were obese and managed with tablets. They got 13 patients to do what was common practice and fashionable 40 years ago. They put the patients on an 800 kcal diet, a diet that has been proven beyond doubt to cause weight loss. Indeed there has never been a report of anyone who can maintain their weight on an 800 kcal diet. Compliance was checked by urinary ketones and weight loss. Eleven of the patients succeeded in finishing the eight week diet and lost as much weight as would be expected from bariatric surgery. Just like what happens following bariatric surgery in patients with type 2 diabetes, the diabetes disappeared and blood pressure and lipids improved. Nothing spectacular so far and the study would not have been worthy of reporting since all this is well known and has been done many times before, as Professor Yki-Jarvinen in her leading article in Diabetologia[3] wrote "the only problem is that in medical school and when I was training as an endocrinologist nobody told me how to get patients to follow such a diet". Only 10% of patients are able to follow dietary restriction advice and only the minority take the exercise treatment. Worse, of those who do succeed 90% relapse. Indeed this is why low calorie diets became unfashionable and large type 2 diabetic trials such as the Steino Hospital trial[4] did not include weight reduction as part of their protocol. The Newcastle group[2] converted an unoriginal and mundane study into a really exciting study by demonstrating that liver fat almost disappeared completely within a week and this was associated with a very large improvement in blood sugar and insulin resistance. The rapidity of improvement was interesting and the significance of the reduction of fat around the beta cell, a new finding of uncertain importance. However a plausible theory is that fat in the vicinity of the beta cell and in particular cholesterol, may be easily oxidised and the release of free radicals contributes to damage to the beta cell. In this regard a gene variant Ckal1, a gene associated with protein translation, has been shown to be very sensitive to oxidation and it is associated with a feeble insulin response[5]. Beta cells have the ability to regenerate and early and intensive reduction in blood sugar has been shown to improve beta cell function. Hyperglycaemia creates a vicious circle-the higher the blood sugar the greater the damage to the beta cell and the greater the damage to the beta cell the higher goes the sugar. Hence the drive to prevent hyperglycaemia by intervention in the pre-diabetes phase and to normalise blood sugar in the early stages of diabetes. The final result of the Newcastle group study that made me and many others sit up and take notice was the demonstration that the beta cell recovered, not partially but completely, and even the first phase insulin release returned to normal so the patients really did reverse their diabetes. This article was of such interest that it made headlines in daily newspapers around the world. Patients and their relatives, perhaps for the first time, really understood the damage diabetes does and gained new hope seeing a goal of reversal of diabetes and the possibility of discontinuation of diabetes medications. Beta trophin has been discovered-a hormone expressed mostly in liver and fat that stimulates beta cell proliferation, expands beta cell mass and improves glucose tolerance in a mouse model[6]. Perhaps an exciting new way to help to reverse diabetes in the future?

The July 2012 edition of the Lancet[7] carried on its cover "Physical inactivity: Worldwide", we estimated that physical inactivity causes 6%-10% of the major non-communicable diseases. Physical inactivity seems to have an effect similar to that of smoking or obesity. Min Lee *et al*[8] examined how much disease could be averted if inactivity were eliminated. Diabetes, as expected, is one of the major diseases the authors looked at. They concluded that not only did physical inactivity account for 6%-10% of the major non communicable diseases but this unhealthy behaviour causes 9% of premature mortality. There is good evidence to demonstrate that overweight or obese children who become obese as adults are at increased risk of diabetes whereas overweight or obese children who became non-obese by adulthood are not[9]. More importantly many studies have shown that educational interventions in physical activity have actually been successful and indeed more successful than interventions for obesity. Heath *et al*[10] in the same issue of the Lancet, examined interventions from around the world and demonstrate that the literature is convincing in demonstrating that behavioural and social approaches are effective. The improvements are seen among people of various ages and from different social groups, countries and communities. The authors make the point that although individuals need to be informed and motivated to adopt physical activity, the public health priority should be to ensure that environments are safe and supportive of health and wellbeing.

Since we know so much about the risk of developing diabetes, it should be possible to have treatment to prevent diabetes in many patients. The diabetes prevention program outcome study[11] has been recently published. This ongoing study demonstrated a clear reduction in diabetes incidence in participants randomly assigned to a lifestyle intervention or metformin during the intervention period. The authors end by stating that their data "support early and aggressive measures for long term prevention of diabetes in people at risk". Intensive lifestyle intervention has been shown to slow the decline in mobility in overweight adults with diabetes[12]. A disappointing result has recently come from the Look AHEAD study[13]. The study was designed to test the hypothesis that an intensive life style intervention for weight loss would decrease cardiovascular morbidity and

mortality in over weight patients with type 2 diabetes. More than 5000 patients took part in the study and the median follow-up of the study was for 9.5 years, weight loss was modest in the intervention group (6% *vs* 3.5% at the end of the study). Alas there was no reduction in the rate of cardiovascular events. The study results are perhaps not surprising in that significant weight reduction is unachievable in most patients but does suggest that we as physicians should accept that most patients are unable to loose weight and should not be made to feel guilty about this. On the other hand to continue to engage the patient in meticulous control of blood pressure, lipids and blood sugar, together with cessation of cigarette smoking, a healthy diet and exercise, are of proven benefit.

Casazza *et al*[14] have written an excellent article entitled "myths, presumptions and facts about obesity". The definition of a presumption was a belief in the absence of supporting scientific evidence; a Myth was defined as a belief persisting despite contradictory evidence. Facts were suppositions backed by sufficient evidence to consider them proven for practical purposes. The authors note that sometimes action is taken by policy makers in the absence of strong scientific evidence "This principle of action should not be mistaken as justification for drawing conclusions". The myths examined were: (1) that small sustained changes in energy intake or expenditure will produce large long term weight changes; (2) Setting realistic goals for weight loss is important otherwise patients will become frustrated and loose less weight; (3) Large rapid weight loss is associated with poor long term weight outcomes as compared with slow gradual weight loss; (4) It is important to assess the stage of diet readiness in order to help patients who request weight loss treatment; (5) Physical education courses in their present form play a part in reducing childhood obesity; (6) Breast feeding is protective against obesity; and (7) A bout of sexual activity burns 100-300 cal for each participant.

A stepwise approach to the management of diabetes has become a fashionable concept in recent years with many published paradigms of the steps which are variable and often contradictory or display so many different stairways that they become very confusing. The first step depends on getting the patient at the very beginning of their path, that is in the pre-diabetes stage but even then they may have already suffered from macrovascular and microvascular damage[15-18]. There is little dissention in advising the lifestyle changes but, should metformin also be used or should one wait and see the effect first of the lifestyle changes? Information on this point is available, for example in the trial by Snehalatha *et al*[19] 2009. There seemed to be no advantage to add metformin to life style changes so perhaps metformin should be reserved for those patients who are unable to adhere to life style changes?

Once diabetes has been diagnosed can one wait and see the result of life style changes or should one aggressively control blood sugar? High glucose is toxic to the beta cell. Exciting new information suggests that the beta cell may dedifferentiate under high glucose attack by causing reduction in a key transcription factor, Fox 01. This dedifferentiation results in the production of inactive proinsulin and an increase in glucagon[20]. Intensive insulin therapy at diagnosis of type 2 diabetes has been shown to reverse diabetes. Weng *et al*[21] studied 382 patients and had divided them into 3 groups. Continuous insulin infusion, multiple injections or oral agents were used to achieve rapid normalisation of hyperglycaemia. Treatment was stopped after normoglycemia was maintained for two weeks. After a year 51% and 44% of the insulin treated patients were in remission where as only 26% of the patients in the oral agent group had gone into remission. The evidence to support early and aggressive treatment for type 2 diabetes has not been widely accepted. The reasons are probably due to a shortage of personnel to manage patients. In my country there is a long waiting list to be seen in a diabetic clinic and general practitioners are usually unhappy about starting insulin. The better understanding of the beta cell pathology of diabetes should persuade physicians to adopt a more urgent approach to diabetes management in the future. A systematic review and meta-analysis on short term intensive insulin therapy in type 2 diabetes gives further support for the ability of this treatment to modify disease progression[22].

BARIATRIC SURGERY

Bariatric surgery for obese type 2 diabetes has been refined over the last few years. Laparoscopic surgery has made operation on morbidly obese patients who have diabetes, and indeed those who do not have diabetes, much safer and very often will reverse the diabetes. The operation has been shown to reduce cardiovascular risk. As with all operations the experience of the surgeon and indeed the surgical unit plays a very important part in outcome. A Cochrane review[23] in 2009 concluded that bariatric surgery is more effective than conventional treatment in achieving and in sustaining weight loss in people with obesity. Improvements in health related quality of life and obesity related co morbidities including type 2 diabetes, dyslipidaemia and sleep apnoea are further benefits. A very good review of the subject has recently been written by Dixon *et al*[24].

Mingrone *et al*[25] in 2012 published a single centre non-blinded randomised controlled trial to examine the difference in outcome between surgery as compared to usual medical therapy. Surgery was either gastric bypass or bilio-pancreatic diversion. At the end of 2 years HbA1c was 6.35% in the gastric bypass group and 4.95% in the bilio-pancreatic-diversion group as compared to 7.69% in the medically treated group. Diabetes remission had occurred in 75% of the gastric bypass group and 95% in the bilio-pancreatic diversion group. No patient in the medical group had reversed their diabetes. There were no deaths and almost no complications in the surgical group[25].

In the same edition of the journal Schauer *et al*[26] evaluated the efficacy of intensive medical therapy as compared to medical therapy plus Roux en Y gastric bypass or sleeve gastrectomy in 150 obese patients with uncontrolled type 2 diabetes. The primary end point was the proportion of patients with a glycated haemoglobin level of 6.0% or less, 12 mo after treatment. Twelve percent of the medical group, 42% in the gastric bypass group and 37% in the sleeve gastrectomy group achieved the primary end point. HbA1c was 7.5% in the medical group 6.4% in the gastric bypass group and 6.6% in the sleeve gastrectomy group. No deaths or life threatening complications occurred[26]. An editorial in the same edition by Zimmet *et al*[27] suggests that the bariatric surgery should not be seen as a last resort. More recently Arterburn *et al*[28] did a retrospective analysis to compare rates of diabetes remission, relapse and all cause mortality amongst severely obese adults with diabetes who underwent bariatric surgery *vs* non-surgical treated individuals. At 2 years the surgery subjects had significantly higher diabetes remission rates 73.7% compared to non surgical subjects with 6.9%. The surgical subjects also experienced lower relapse rates with no higher risk of death[28].

NEW INSULINS FOR TREATMENT OF TYPE 2 DIABETES

Many different regimes have been proposed and indeed are in use for the treatment of type 2 diabetes when life style and metformin have failed to control hyperglycaemia. A three year efficacy of complex insulins in type 2 diabetes demonstrated that the addition of a basal or prandial insulin based regimen to oral therapy had better diabetic control than those who added a biphasic insulin regimen[29]. My own feeling is that, as so many patients with type 2 diabetes don't increase their blood sugars overnight, attention should be paid to controlling the post evening meal rise in blood sugar so that the patient goes to bed with a normal blood sugar, long acting insulins being reserved for those patients in whom blood sugars rise overnight. To me it doesn't make sense to give a basal dose of a long acting insulin pre bed with the risk of overnight hypoglycaemia to a patient whose blood sugar has not been shown to rise overnight. Insulin degludec is almost identical to human insulin but with the last amino acid deleted from the B chain and addition of a glutamyl link from LysB29 to a hexadecanoic fatty acid[30]. Two phase 3 studies were reported recently[31,32]. In the first study type 1 diabetic patients (472 subjects) were subjected to insulin degludec and 157 to glargine insulin[31]. Although there was no difference in HbA1c at the end of the study and no difference in overall, confirmed hypoglycaemia; overnight hypoglycaemia was 25% less in the insulin degludec and of course nocturnal hypoglycaemia is what many patients fear most. The second study Garber *et al*[32] reported the effect of the new insulin in type 2 diabetic patients *vs* insulin glargine. Again after 1 year there was no difference between the 2 groups in HbA1c. Overall hypoglycaemia was a little less in the insulin degludec group and nocturnal hypoglycaemia was also a little lower (1.4 *vs* 1.8 episodes per patient-year exposure). The authors conclude that the newer basal insulins with lower hypoglycaemia events may allow more intensive blood sugar lowering treatment. From the results presented in their paper, insulin degludec does not seem to be the answer. An editorial by Tahrani *et al*[33] in the same edition, ends by saying that insulin degludec is not a revolution but an evolution of insulin therapy for patients with both type 1 and type 2 diabetes.

SODIUM GLUCOSE CO-TRANSPORT-2 INHIBITORS

Glycosuria occurs when the blood glucose reaches a threshold of about 10 mmol/L. However some people will excrete glucose at much lower levels of blood glucose (renal glycosuria). The discovery that glucose is transported across the proximal tubule membrane by sodium/glucose co-transport 2 (SGLT2) and that a naturally occurring polymorphism of the gene causes renal glycosuria, paved the way for the development of SGLT2 receptor inhibitors as a way of promoting renal glucose excretion and therefore calorie loss and reduction of blood sugar. Two drugs have undergone clinical trials dapaglifozin and canaglifozin and have been the subject of a meta analysis by Clar *et al*[34]. The drugs both result in blood glucose reduction of about 0.5%-1% with some weight loss. Urinary and genital infections were more common. Hypoglycaemia did not occur any more frequently that placebo. The results of the Cantata-SU trial have recently been published[35]. The trial was a 52 wk study in type 2 diabetes with patients who were inadequately controlled with metformin. Canagliflozin was compared to Glimepiride. 1452 patients were randomised in a phase 3 non-inferiority, double blind, randomised trial. Three hundred mg of Canagliflozin reduced HbA1c from a mean of 7.8% to 6.9% (mmol/L) a reduction of 0.9%. Hypoglycaemia was less common on Canagliflozin and there was a 4 kg reduction in weight with a small reduction in blood pressure. There was a 0.25 increase in LDL cholesterol but also a slight, 0.1% increase in HDL cholesterol and a very slight reduction in triglycerides also of 0.1%. Genital mycotic infections occurred in 8% in men and 14% in women on the 300 mg dose. The study suggests that the benefit of the drug is a useful reduction in HbA1c and weight reduction. The blood pressure reduction is also of benefit but the rise in LDL might be a worry and the mycotic genital infections and urinary tract infections might make the drug unacceptable to many patients who may have presented with these problems when first diagnosed. An editorial in the Lancet where the results were published is entitled "SGLT2 inhibitors for diabetes: turning symptoms into therapy" and makes the point that the place of this class of drugs in the treatment of type diabetes is still to be decided[36]. There has been concern about breast and bladder cancer as well

as long-term cardiovascular adverse effects also making surveillance mandatory. Another recently published study comparing canagliflozin with placebo and sitagliptin produced similar results[37]. A randomised, blinded, prospective Phase 111 study on dapagliflozin as monotherapy in drug naive Asian patients with type 2 diabetes found that with the 10 mg dose HbA1c had fallen from a mean of 8.26% to 7.15% as compared to a fall of only 0.29% for placebo(a difference of 0.82%) Genital infections occurred in 4.5% of patients and Urinary tract infections in 5.3%[38].

The role of these drugs in the treatment of type 2 diabetes is not clear at present but the lack of risk of hypoglycaemia and the weight reduction suggest that there is a place for them in certain patients who are inadequately controlled and in whom an extra 0.5% or more reduction in blood sugar would be of benefit in bringing the patient into the acceptable blood sugar range.

METFORMIN

The reason for metformin as first line pharmacological treatment is based on many studies suggesting that metformin is weight neutral or associated with very modest weight loss as compared with sulphonylureas which cause slight weight gain initially. Also, in experimental conditions reperfusion after myocardial infarction is reduced by sulphonylureas. As long ago as 1971 the University Group Diabetes Program[39] showed that tolbutamide, a first generation sulphonylurea, was associated with an increased cardiovascular risk in diabetes. The UKPDS trial[40] suggested that metformin has a protective effect on mortality. Roumie *et al*[41] examined the comparative effectiveness of sulphonylurea and metformin monotherapy on cardiovascular events in type 2 diabetes mellitius. This was a very large retrospective cohort study examining cardiovascular outcomes. The crude rates of composite outcome were 18.2 per 1000 person years in the sulphonylurea users and 10.4 per 1000 person years in the metformin group. A wonderful editorial in the same edition of the Annals of Internal Medicine by Nissen[42] entitled "Cardiovascular effects of Diabetes Drugs; Emerging from the dark ages", likens the dark ages after the fall of the Roman Empire to the time between the University Group Diabetes Program in 1972[39] which showed that treatment for diabetes with phenformin or tolbutamide was associated with increased cardiovascular risk, and 2012. The article explains why there is still uncertainty about the effect of sulphonylureas and cardiovascular events. Nissen[42] suggests that the study is hypothesis generating rather than definitive and that high quality evidence is still missing "Continued darkness is not an acceptable option" he concludes.

INCRETINS

It has been known for many years that intravenous glucose will not stimulate insulin secretion to the same extent as a similar glucose load given orally. It was discovered that hormones secreted from the intestine in response to a glucose load had the ability to release glucose from the pancreas. These hormones were called incretins and they are responsible for at least 50% of insulin secretion following a meal. In 1971 a peptide was isolated from the intestine which had the ability to inhibit gastric acid secretion and was therefore called gastric inhibitory polypeptide (GIP)[43]. GIP was later found to stimulate insulin secretion. What was very interesting was that GIP would only stimulate insulin secretion in the presence of high blood sugar. This finding has implications in treatment terms since drug that only works with high blood sugar would be much less likely to cause hypoglycaemia. Patients, their families and of course doctors and other health care professionals all fear hypoglycaemia. Garber[44] refers to the many hospital visits caused by hypoglycaemia and suggests that minimisation of hypoglycaemia should be a goal for treatment of type 2 diabetes. I would certainly agree. In a survey insulin accounted for 13.9% of overall admissions to hospital from adverse drug reactions and oral anti-diabetic drugs 10.7%[45].

Another incretin was discovered in 1985 and called glucagon-like peptide-1 (GLP-1)[46]. This hormone was also dependent on high blood sugar level for full action. Both GIP and GLP-1 act by binding to specific receptors and so release insulin. GLP-1 has another action, it inhibits gastric emptying and this has been of benefit in the treatment of diabetic patients because the feeling of satiety leads to weight reduction. Another beneficial effect of the reduction in rate of gastric emptying is to delay absorption of food, a mechanism which improves blood sugar excursion. GLP-1 also regulates appetite and food intake through its effect the hypothalamus. A recent review of the effects of GLP1 on appetite and body weight with a focus on the central nervous system has been published[47].

GLP-1 agonists have been shown to stimulate B cell growth in animals and cell cultures. In humans it is less clear if these drugs can improve insulin output by regenerating the B cell. It seems less likely that the dipeptidyl peptidase (DPP)-4 inhibitors could also have an effect on B-cell re-growth. However an abstract presented at the Annual American Diabetes Association meeting in 2010 suggested that linagliptin was able to restore beta cell function in human isolated islets[48]. Vildagliptin has also been shown to improve beta cell function and glucose tolerance but also to improve the extensive peri-insulitis found in the mouse model examined[49].

A very interesting effect of GLP-1 analogue therapy has been described in obese type 2 diabetic patients. The investigators found a reduction in inflammatory macrophages and a reduction in inflammatory cytokines together with an increase in the adipokine adiponectin. The researchers had previously described a case of psoriasis that was greatly improved by GLP-1 agonist therapy[50]. The new study does suggest an important beneficial effect of GLP-1 analogue therapy that needs further inves-

tigation[51]. A good review on the extrahepatic effects of GLP-1 receptor Agonists has just been published[52].

DEVELOPMENT OF GLP-1 FOR THE TREATMENT OF DIABETES

Exenatide is a GLP-1 receptor agonist. It is a 39 amino acid peptide produced in the saliva glands of the Gila monster lizard[53] it has 53% amino acid homology to full length GLP-1 and it binds with greater affinity than GLP-1 to the GLP-I receptor in GLP-1 receptor expressing cells[54]. DPP-4 cleaves peptides and is responsible for the rapid breakdown of GLP-1. DPP-4 does not denature exenatide because of the slight amino acid differences and in human studies the half life ranges from 3.3 to 4 h[55]. Exenatide (Eli Lilly) is now in clinical use in many countries for the treatment of diabetes. It must be given an hour before meals on a twice a day basis. Many trials have reported that the drugs cause about a 1% reduction in HbA1c and reduction in body weight of 5.3 kg at the end of 3 years of treatment[56]. The dropout rate is about 20%, many patients refusing treatment because of nausea.

EXENITIDE

Attempts have been made to prolong the action of exenatide using a polylactide glycolide microsphere suspension so that the drug can be given weekly. Kim *et al*[57], in a randomised placebo-controlled phase 2 study examined the effect of exenatide long acting release, a long acting release exenatide formulation, found that a weekly dose for 15 wk in patients with type Ⅱ diabetes resulted in a 1.4% reduction in HbA1c, suggesting that once a week formulation may be as good as, if not better than, twice daily injections of exenatide. In particular there were no dropouts in the trial due to adverse events. Liraglutide is a long acting GLP-1 analogue with attachment of a C-16 free fatty acid derivative. The free fatty acid derivative promotes non-covalent binding of liraglutide to albumen thereby increasing plasma half life. A recent study comparing liraglutide once a day with exenatide twice a day found that liraglutide improved HbA1c significantly more (-1.12% *viz* -0.79%) and was generally better tolerated[58]. The study has demonstrated that glycaemic improvement and weight reduction are independent of each other. This fits in with other studies which suggest that the weight loss is not, in itself, the cause of the improved blood sugar control[59].

In a recent paper Derosa *et al*[60] examined the effect of exenatide on beta cell function. The authors used the homeostasis model assessment beta cell function index as well as assessing pro-insulin and insulin with arginine stimulation under clamp conditions. The results suggested that beta cell function was improved by exenatide. However a caveat, HbA1c was significantly better after the 12 mo of exenatide as compared to placebo. It is well known that hyperglycaemia is toxic to the beta cell hence the improved glucose might have been responsible for the beta cell improvement rather than the drug itself. Bunck *et al*[61] showed similar results compared to glargine. In their study combined glucose and arginine stimulated C peptide secretion was 2.46 fold greater after 52 wk of exenatide treatment compared with insulin glargine treatment with a non significant (P = 0.55) 0.8% reduction in HbA1c as compared to a -0.7% reduction in the glargine group. Four weeks after cessation, the beta cell function returned to pre treatment levels.

Exenatide, was compared to glimepiride in patients who were not controlled on metformin alone[62]. About 1000 patients were divided into 2 groups and studied on average for 2 years although some went on for 42 mo. At the end of 3 mo both groups had decreased HbA1c from around 7.4% to 6.8% but by 36 mo the glimperamide group had gone back to a HbA1c of more than 7.3% whereas the exenatide group, although increasing their HbA1c slowly over the 3 years, was significantly lower at a level of just over 7.2%. Body weight fell in the exenatide group by 3.32 kg and rose in the glimperamide group by 1.15 kg. Systolic blood pressure (BP) decreased in the exenatide group by 1.9 mmHg with no change in the Glimpereride group. Less patients in the exenatide group experienced a hypoglycaemic episode. In the first 6 mo 49 patients in the exenatide group discontinued mostly due to gastrointestinal side effects as compared to 17 in the glimepiride group (P = 0.001) Buse *et al*[63] examined whether twice daily exenatide injections reduced HbA1c levels more than placebo in patients receiving Glargine insulin. HbA1c decreased by 1.74% in the exenatide group as compared to 1.04% in the placebo group over a 30 wk period. Hypoglycaemia was similar in the 2 groups and 13 treatment patients and 1 placebo recipient discontinued the study because of adverse events, nausea and vomiting being the main problems.

LIRAGLUTIDE

At the beginning of 2012 the FDA approved the marketing of extended release exenatide (Bydurion). The drug is given weekly by injection. Liraglutide is a human GLP-1 analog given by once daily injection with a good safety record and HbA1 lowering effect similar to the other GLP-1 agonists. A 2-year report on safety, tolerability and sustained weight loss over 5.2 years with once daily liraglutide has been published[64]. Two hundred and sixty eight of 398 people who entered the extension of the original 20 wk trial completed 2 years. Weight loss was 7.8 kg from screening and was maintained. There were improvements in BP and lipids. Patients with diabetes however were excluded from taking part in this trial. The Duration Trial 6[65] reported on a study comparing daily liraglutide to weekly extended release exenatide. This was a 26 wk trial with more than 400 patients in either arm. Liraglutide was associated with a greater change in HbA1c (-0.48% *viz* 1.28%). Nausea was more common in the liraglutide group (21% *viz* 9%) and also vomiting (11% *viz* 4%) 5% of patients allocated to liraglutide discontinued the treatment as compared to 3% allocated to exenatide because

of adverse events. The results suggest that the patient might be allowed to choose whether to have a drug which is injected daily but with no diluting procedure before the injection or a weekly injection with less blood sugar lowering effect but less side effects. Non-alcoholic steatosis has become a problem in type 2 diabetes. The LEAN study is currently examining whether liraglutide will improve non-alcoholic steatohepatitis outcome[66].

LIXISENATIDE

Lixinitide is another potent, selective, once daily GLP-1 agonist. A randomised placebo controlled double blind trial examined lixisentide daily injection in Asian patients with type 2 diabetes insufficiently controlled on basal insulin with or without sulphonylureas[67]. This was a 24 wk study. These patients were not obese body Mass Index 25.3 kg/m^2. Eighty-two percent of patients reached and stayed on the maintenance dose of lixisenatide (20 μg once a day). There was a significant reduction in HbA1c compared to controls. The difference at the end of the trial was 0.88%. There was no significant change in weight compared to controls. The incidence of serious side effects were similar in both groups. Two patients in the lixisenatide group experienced cerebrovascular infarction. Forty-two percent of study drug patients experienced hypoglycaemia as compared to 24% on placebo. Fonseca *et al*[68] examined efficacy and safety of once daily lixisenatide at different doses. HbA1c was reduced by 0.66% compared to placebo. Postprandial and fasting blood sugars were significantly lower in the treatment group. In a study by Kapitza *et al*[69] lixisenatide once daily was compared to liraglutide once daily in patients with type 2 diabetes insufficiently controlled on metformin. This was only a 28 d study but the results showed that liraglutide controlled fasting blood glucose better than lixisenatide but postprandial blood sugar was better controlled by lixisenatide. A review discussing the place of this GLP-1 agonist as an add on therapy to basal insulin has recently been published[70].

TASPOGLUTIDE

Ipsen Roche had another GLP-1 analogue under review called taspoglutide. This is a GLP-1 analogue which has a prolonged action and is in phase Ⅱ trials. The drug has been shown to improve diabetes control and lowers body weight in subjects with diabetes. In a study involving once a week injections in 306 type 2 diabetic subjects who were already on Metformin, 8 wk treatment was associated with a reduction in HbA1c. The highest dose gave an HbA1c reduction of 0.9% and a weight reduction of 1.9 kg as compared to placebo. Nauck *et al*[71] report on a 24 wk study using a 10 mg or a 20 mg dose of Taspoglutide, comparing once a week dosing to daily glargine insulin. One thousand and forty-nine patients were randomised into 3 groups. Withdrawal rates were 21% for each of the Taspoglutide groups and 9% for the glargine group. HbA1c of < 53% was achieved in 39.47% and 32% receiving Taspoglutide 10 mg, 20 mg and HbA1c < 48 in 18%, 24%, and 14% of patients or glargine insulin respectively. Lower fasting blood sugars were achieved by glargine insulin. Serious hypersensitivity reactions occurred in 2 patients on Taspoglutide. However confirmed hypoglycaemia was less with the study drug (0.3%, 0.9% *viz* 3.1%) and weight loss was greater on Taspoglutide (-3.3 and -4.1 kg). Withdrawals due to adverse effects occurred in 9%, 13% on Taspoglutide and in 1% on the glargine insulin. An addendum to the paper states that Roche has now stopped the development of the drug. Ibsen is currently pursuing further investigations. Rosenstock *et al*[72] examined the fate of Taspoglutide once a week *vs* Exenatide for type 2 diabetes. The doses used were again 10 mg or 20 mg as compared to twice daily exenatide 10 μg. Reduction in HbA1c was -1.24 with 10 mg and -1.31 with the 20 mg as compared to exenatide from a starting HbA1c of 8.1%. Withdrawals were higher in the study drug patients and the authors conclude that even though Taspoglutide caused lower blood sugars the level of side effects was unacceptable.

Albiglutide is a long acting subcutaneous albumen-based fusion of GLP-1[73]. In February 2009 Glaxo SmithKline (GSK) began phase 3 studies in type Ⅱ diabetes. Albiglutide is a GLP-1 mimetic generated by genetic fusion of a DPP-4-resistant GLP-1 dimer to human albumin[74]. The formulation was originally developed by Human Genome Sciences (HGS) and named Albugon, GSK having bought the drug in 2004 for all human therapeutic and prophylactic applications of Albiglutide. In 1999 Centeon (now Aventis Berring) granted Principia (now HGS) world wide rights to its recombinant fusion proteins and its related yeast technologies[75].

ANTIBODIES TO GLP-1 AGONISTS

Therapeutic proteins/peptides with structural similarity to endogenous proteins/peptides often have unwanted immunogenicity. Antibodies to the GLP-1 agonists have been described and may inhibit the action of the agonist. The role of antibody formation to the various agonists on the market at present are uncertain. A study by Buse *et al*[76] in 2011 suggested that antibodies to liraglutide did not inhibit efficacy however antibodies to exenitide, if they were high, was associated with a smaller HbA1c reduction. Antialbiglutide antibodies developed in 2.5% of patients in an 8 wk trial.

GLP-1 AND THE CARDIOVASCULAR SYSTEM

Endothelial dysfunction is a common finding in diabetes and an early marker of atherosclerosis. GLP-1 has been shown to improve endothelial dysfunction[77,78]. GLP-1 exerts a cardio-protective effect against ischaemic damage and heart failure. Diabetes is associated with an increased risk of atherosclerosis and myocardial infarction.

Ischaemic preconditioning is a protective mechanism by which the heart may protect itself from prolonged ischaemia. The University Group Diabetes Programme report[39], more than 40 years ago, suggested that tolbutamide might increase myocardial infarction and mortality. Glibenclamide has been shown to affect ischaemic preconditioning but trials have not shown beyond doubt that it is associated with increased myocardial infarction. However drugs that inhibit the K ATP channel opening, such as glibenclamide, are related to loss of ischaemic preconditioning[79-81]. GLP-1 receptors are found in the heart. Increased glucose uptake by the cardiac myocyte is beneficial in protecting the heart from ischaemia changes[82]. Studies in situ and *ex-vivo* suggest a beneficial effect on the heart muscle when under ischaemic stress. Bao *et al*[83] examined the effect of albiglutide in rats after myocardial ischaemia reperfusion injury. They measured cardiac glucose uptake and cardiac metabolic flux. They found enhanced glucose uptake and reduced myocardial infarct size and improved cardiac function. It has yet to be shown if this effect also occurs in humans and if myocardial infarct size and mortality will be reduced by GLP-1 agonists. DPP-4 inhibitors have been less well studied in cardiac ischaemic preconditioning. In a study by Rahmi *et al*[84] rapaglinide, a sulphonylurea like drug, inhibited ischaemic preconditioning as measured by stress testing in patients with type 2 diabetes who already had evidence of coronary atherosclerosis. Vildagliptin, a DPP-4 inhibitor, did not alter preconditioning in 72% of patients whereas 83% of the repaglinide patients had ischaemia earlier in their stress test.

GLP-1 AND THE PANCREAS

Pancreatitis has been described in patients using GLP-1 agonists. A report in 2010 stated that 8 cases during clinical development and 36 post marketing reports are available[85]. A recent report[86] examined a large United States health insurance claims database and could find no increased risk of acute pancreatitis using twice daily exenatide. However there were several limitations to the study and it was a pity that other GLP-1 agonists were not investigated at the same time but the study was funded by Amylin and Eli Lilly. Stimulation of GLP-1 receptors that are found in the exocrine pancreas might lead to overgrowth of the epithelial cells in the small ducts causing pancreatitis through obstruction. A worry has been raised that GLP-1 agonists may induce metaplasia and premalignant changes[87,88].

GLP1 AND THE THYROID

The thyroid contains GLP1 receptors and Gier *et al*[89] also found coincident immunoreactivity for calcitonin and GLP-1 receptors in both medullary thyroid carcinoma and C cell hyperplasia. C cell carcinoma of the thyroid has been seen in animals dosed with GLP-1 agonists and can be explained by the finding of GLP-1 receptors in the thyroid[89]. GLP-1 receptor immuno-reactivity was also found in 18% of papillary thyroid carcinoma. The authors speculate on the consequences of long term stimulation of these GLP-1 receptors. They suggest that prospective studies need to be done to exclude an increase in papillary and medullary carcinoma in the thyroid.

DPP-4 INHIBITORS

These drugs act by inhibiting the enzyme that breaks down GLP-1, thus increasing the level of GLP-1 in the blood stream. They are however not able to raise the GLP-1 levels to levels found after injection of GLP-1 agonists and therefore their hypoglycaemic efficacy is less than that of GLP-1 agonists. Sitagliptin, vildagliptin, saxagliptin and linagliptin have already been approved in the United States and in Europe. An excellent systematic review and meta-analysis has been published in the British Medical Journal in 2012[90]. Compared with metformin, DPP-4 inhibitors were associated with a smaller decline in HbA1c and a lower chance of attaining a HbA1c goal of less than 7%. As a second line treatment DPP-4 inhibitors achieved a smaller decline in HbA1c than the other hypoglycaemic drugs. There was however, no significant difference in attaining an HbA1c of less than 7% when compared to sulphonylureas. They were less effective in lowering body weight when compared to metformin. When added to metformin they had a favourable weight profile compared to metformin and sulphonylureas or pioglitazone but not when compared to GLP-1 agonists. Hypoglycaemia was less common when a DPP-4 inhibitor was added to metformin as compared to a sulphonylurea added to metformin. There is evidence to suggest that the DPP-4 inhibitors are more effective in lowering glucose in Asians than non Asians[91]. A one year follow up of DPP-4 inhibitors *vs* sulfonylureas on top of metformin has been published recently[92]. Patients with prior metformin therapy received a dual combination of metformin with either DPP-4 inhibitor or sulfonylureas. There was no significant difference in either body weight or HbA1c. Hypoglycaemia was significantly less in the patients taking DPP-4 inhibitors. These patients had significantly less transitory cerebral ischaemic attacks whereas other cardiovascular events were of borderline significance.

There are 6 DPP-4 inhibitors (*e.g.*, Sitigliptin, Linagliptin, Vildagliptin, Alogliptin, Saxagliptin, Teneligliptin) on the market minor variation in their chemical composition have not been translated to particular benefit although it should be noted that linagliptin is mostly excreted in pathways other than the kidney and hence dosage does not have to be reduced in moderate renal failure.

Vildagliptin, a DPP-4 inhibitor which increases circulating GLP-1 levels, has been shown to ameliorate the deposition of amyloid beta and tau phosphorylation in a streptozotosin induced animal model of diabetes[93]. A study by Omar *et al*[94] using a high fat diet induced obesity model in mice of advanced age has demonstrated that Vildagliptin confirms other rodent models of diabetes in preserving beta cell mass mainly through inducing beta cell proliferation and reducing beta cell apoptosis[94-96].

Omar *et al*[94] found that Vildagliptin improved glucose secretion in response to oral glucose. Beta cell area was not significantly altered by Vildagliptin treatment in these mice but peri insulitis was prevented by Vildagliptin. Sitagliptin has also been shown to protect against amyloid associated beta cell loss but its effect was not different to that of Metformin[97].

The binding modes of these drugs has recently been investigated[98]. Based on their binding sites the authors divided the drugs into 3 categorise, Vildagliptin and Saxagliptin, Alogliptin and Linagliptin, Sitagliptin and teneligliptin. It is not clear whether these different binding modes have clinical relevance but may help in the development of better inhibitors in the future. Unlike GLP-1 agonists the DPP-4 inhibitors do not pass the blood brain barrier and have no effect on satiety, nor do they effect gastric emptying. Although the different DPP-4 inhibitors have some differences including potency, half lives and metabolism there does not seem to be any meaningful difference in their ability to lower blood sugar and this is probably why there are virtually no head to head studies (one head to head study showed no difference between saxagliptin and sitagliptin when combined with metformin[99]. A good review of the differences has been written by Capuano *et al*[100]. Most of the DPP-4 inhibitors can be administered once daily but Vildagliptin needs to be given twice daily. Saxagliptin is mainly metabolised by CYP3A4/5 isoforms to a major active metabolite 5-saxahydroxygliptin. It is suggested that the dosage of saxagliptin be modified if co administration with CYP3A4/5 inducers such as rifampicin or inhibitors such as ketoconazole.

SITAGLIPTIN

Insulin glargine *vs* sitagliptin another DPP-4 inhibitor was studied by Aschner *et al*[101]. About 250 patients in each group were studied for more than 6 mo. At the start patients were already on metformin which was continued during the study. HbA1c was significantly lower in the glargine group. There were more hypoglycaemic episodes and slight weight gain in the glargine group where as there was slight weight loss in the Sitagliptin group. A recent study compared the effect of sitagliptin or glibenclamide in addition to metformin and pioglitazone on glycaemic control and beta cell function[102]. Body weight reached was lower with sitagliptin. Fasting plasma insulin and homeostasis model assessment of insulin resistance with glibenclamide were significantly increased with glibenclamide and decreased with sitagliptin. Sitagliptin did not change the homeostasis model assessment of beta cell function but the value was significantly increased by glibenclamide. Both glibenclamide and sitagliptin increased C-peptide.

VILDAGLIPTIN

A 24 wk study in elderly patients was recently published[103]. The study investigated the feasibility of setting and achieving individualised targets over 24 wk for elderly patients (over 70 years of age with type 2 diabetes). The patients who were treated with vildagliptin achieved a 0.6% reduction in HbA1c from a baseline of 7.9% as compared with placebo. There were no tolerability issues as compared to placebo, hypoglycaemic events were 2.2% in the vildagliptin arm and 0.7% in the placebo arm. Individualising goal HbA1c is thought to be appropriate particularly in the frail elderly[104]. The benefit of reducing HbA1c by less than 1% in this age group is uncertain. There seems no doubt that in the frail elderly hypoglycaemia is a very serious threat to health[105,106]. Macrovascular disease/events seem to respond better to blood pressure and lipid interventions than to blood sugar lowering at least in the short term[107] but microvascular damage and retinopathy prevention, particularly in patients who already have significant damage, should make the Physician consider carefully the probable benefit of tighter blood sugar control. Under these circumstances one might not choose a DPP-4 inhibitor since they work better in the higher blood sugar range and are less likely to result in the achievement of a HbA1c of 6.5% (48 mmol/L). The efficacy and safety of vildagliptin in patients with type 2 diabetes inadequately controlled on Metformin and sulphonylurea suggests that a mean HbA1c of 8.75% can be improved by about 0.75% as compared to placebo[108]. It is such a pity that the GLP-1 agonists work best at high HbA1c levels and are less effective in reduction of HbA1c as the HbA1c gets near to target. However in this trial 25% more patients reached a target of 7% as compared to controls(38.6% *viz* 13.9%).

SAXAGLIPTIN

The 4-year safety of saxagliptin has recently been published[109]. No new safety issue findings appeared during the 4 years of treatment alone or with metformin and hypoglycaemia did not increase the risk of hypoglycaemia. The cardiovascular safety of diabetic drugs continues to raise concern[109]. Saxagliptin was examined by Scirica *et al*[110]. They randomised 16492 patients with type 2 diabetes who had a history of or who were at risk for cardiovascular events, to receive Saxagliptin or placebo and followed them for a median of 2.1 years. The HbA1c at the beginning of the study was 8.0% and at the end of the study the HbA1c in the Saxagliptin arm had decreased to 7.5% and the placebo arm to 7.8%. A surprising finding was that more patients in the Saxagliptin group were hospitalised for heart failure but otherwise the cardiovascular end point results were similar between the two groups. Hospitalisation for hypoglycaemia occurred infrequently and was similar in the two groups but significantly more patients in the saxagliptin group reported at least one hypoglycaemic event. Thus this 2-year study gives little support for the use of saxagliptin in these patients.

LINAGLIPTIN

Linagliptin is a once a day oral DPP-4 inhibitor. It is an

orally active small molecule which was licensed in United States in 2011. It is mostly excreted in the faeces and there are no clinically relevant alterations in linagliptin pharmokinetics resulting from renal or liver impairment[111]. A recent study has confirmed that renal impairment has no clinically relevant effect on the long term exposure of linagliptin in patients with type 2 diabetes[112].

A 2-year efficacy and safety study of linagliptin compared to glimepiride in patients inadequately controlled on metformin was reported recently[113]. More than 1400 patients were divided into two groups. HbA1c at the end of the study was similar in the two groups but there was less hypoglycaemia and there were significantly less cardiovascular events (1 *vs* 2). Hypoglycaemia is not usually a problem in the treatment of type 2 diabetes but recently has been suggested to be a therapeutic concern. The efficacy and safety of Linagliptin in subjects with type 2 diabetes was analysed by Del Prato *et al*[114]. Pooled analysis of data from 2258 subjects in 324 wk phase 3 studies. Oral linagliptin or placebo as monotherapy added on to metformin or added on to metformin plus a sulphonylurea were the treatments investigated. Although linagliptin was effective the patients had a mean HbA1c of 9.0% and the level of HbA1c only dropped to 8.3% still unacceptably high for many patients. DPP-4 inhibitors unfortunately work less well the lower the starting HbA1c[102]. A study of linagliptin in patients aged over 70 years found that HbA1c was lowered by 0.64% from 7.8% to 7.2% with a safety profile similar to placebo. Whether long term studies in this age group will show benefit in measurable outcome is speculative at this time.

ALOGLIPTIN

Alogliptin seems to have much the same characteristics as the other DPP-4 inhibitors on the market, A useful review has recently been published[115]. Another large study specifically looking at cardiovascular disease in type 2 diabetic patients has been reported[116]. More than 5000 patients who had type 2 diabetes and either an acute myocardial infarction or unstable angina requiring hospitalisation within the previous 15 to 90 d received allogliptin or placebo in addition to existing antidiabetic and cardiovascular drug treatment. HbA1c at the start of the trial was 8.0% and at the end of the study had come down to 7.7% as compared to 7.97% in the placebo group. Hypoglycaemia was similar in the two groups. Again this large study makes one question the value of the addition of the DPP-4 inhibitor which was associated with such a modest drop in HbA1c.

TENELIGLIPTIN

Teneligliptin is another DPP-4 inhibitor which has been recently reviewed[117].

DPP-4 INHIBITORS AND THE HEART

GLP-1 receptors, which are found in the heart increase glucose uptake by the cardiac myocyte is beneficial in protecting the heart from ischaemia changes[118]. Matsubara *et al*[119] examined 44 patients with coronary artery disease and uncontrolled diabetes (HbA1c > 7.4%). Sitagliptin or aggressive conventional treatment was compared after 6 mo. Endothelial function was significantly improved in the sitagliptin group with no difference in fasting blood sugar at the end of the trial but a reduction in HbA1c of 0.6% in each group. C-reactive protein (CRP) reduced significantly in the sitagliptin group with a significant correlation between the CRP and the vascular reactivity but not with HbA1c.

DPP-4 INHIBITORS AND THE PANCREAS

Butler *et al*[120] examined the pancreata of 7 individuals treated with sitagliptin and 1 with exenatide compared with 12 individuals with type 2 diabetes treated with other agents, and 14 non-diabetics. There was an increase in the number of pre-malignant lesions and marked alpha cell hyperplasia with glucagon expressing micro adenomas and a glucagon expressing neuroendocrine tumour in one of the eight. Because the number of diabetics who were not on treatment with DPP-4 based therapy were so few the evidence is insufficient for alarm but the evidence for caution and vigilance in the next number of years is clear and persuasive.

Sero negative polyarthropathy has been recorded with the use of DPP-4 inhibitors. Three patients were described by Crickx *et al*[121] and one case by Ambrosio *et al*[122]. The acute arthritis is not perhaps surprising since DPP-4, also named CD 26 is expressed on many cells involved in the immune process.

CONCLUSION

New treatments for diabetes are coming on line but prevention and treatment of obesity through increased exercise and reduced calorie intake still seems the best option in most patients with type 2 diabetes. Those with insulin deficiency have new options which are exciting as they demonstrate new approaches to treatment but their glucose lowering effects are modest and mostly most effective when blood sugars are high thus of less use when blood sugars are near to, but not at, target in spite of a combination treatment.

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P- Reviewer: Apikoglu-Rabus S, Conteduca V, Kumar KVS
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
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ISBN 978-0-9861420-0-0

Acute effects of physical exercise in type 2 diabetes: A review

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Author contributions: All authors contributed in all phases of the study: search articles, literature review, writing and reviewing of the manuscript.
Supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)
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Received: June 10, 2014 Revised: July 9, 2014
Accepted: July 25, 2014
Published online: October 15, 2014

Abstract

The literature has shown the efficiency of exercise in the control of type 2 diabetes (T2D), being suggested as one of the best kinds of non-pharmacological treatments for its population. Thus, the scientific production related to this phenomenon has growing exponentially. However, despite its advances, still there is a lack of studies that have carried out a review on the acute effects of physical exercise on metabolic and hemodynamic markers and possible control mechanisms of these indicators in individuals with T2D, not to mention that in a related way, these themes have been very little studied today. Therefore, the aim of this study was to organize and analyze the current scientific production about the acute effects of physical exercise on metabolic and hemodynamic markers and possible control mechanisms of these indicators in T2D individuals. For such, a research with the following keywords was performed: -exercise; diabetes and post-exercise hypotension; diabetes and excess post-exercise oxygen consumption; diabetes and acute effects in PUBMED, SCIELO and HIGHWIRE databases. From the analyzed studies, it is possible to conclude that, a single exercise session can promote an increase in the bioavailability of nitric oxide and elicit decreases in postexercise blood pressure. Furthermore, the metabolic stress from physical exercise can increase the oxidation of carbohydrate during the exercise and keep it, in high levels, the post exercise consumption of O_2, this phenomenon increases the rate of fat oxidation during recovery periods after exercise, improves glucose tolerance and insulin sensitivity and reduces glycemia between 2-72 h, which seems to be dependent on the exercise intensity and duration of the effort.

Key words: Metabolic diseases; Hypertension; Nitric oxide; Blood glucose; Oxygen consumption

Core tip: Physical exercise is one of the best kinds of non-pharmacological treatments to prevent and control type 2 diabetes (T2D), being recommended by important medical associations, such as American College of Sports Medicine and the American Diabetes Association. In the literature, studies about the effects of a single exercise session on the population, its changes in blood pressure, glycemia, carbohydrate oxidation, fat oxidation, increase in nitric oxide and others are increasing exponentially. In this review, we report the most recent and important findings in the literature about the ef-

fects of acute exercise in T2D.

Original sources: Asano RY, Sales MM, Browne RAV, Moraes JFVN, Coelho Júnior HJ, Moraes MR, Simões HG. Acute effects of physical exercise in type 2 diabetes: A review. *World J Diabetes* 2014; 5(5): 659-665 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i5/659.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i5.659

INTRODUCTION

Physical exercise, along with a proper diet are central factors in the prevention and control of diabetes mellitus (DM), since their effects include appropriate values of blood pressure, glycemia and lipidemia[1]. Several studies have shown the efficiency of exercise programs in the control of DM, being suggested as one of the best types of non-pharmacological treatments to the population in question[2-5]. Aerobic, resistance or combined exercise programs can help in the control of glycemia of diabetes mellitus type 2 (T2D), mainly by the increase of the need of glucose consumption by skeletal muscle in activity and the hypoglycaemic effect after exercise has been performed[1,6-9].

Currently, the guidelines to physical exercise prescription by the American College of Sports Medicine and American Diabetes Association to T2D provide general information, such as exercise daily, accumulate 150 min of exercise in a moderate intensity or 75 min of high intensity exercise per week; resistance exercises should be included at least 2-3 times per week[1]. On the other hand, despite the advances made in discovering the effects of exercise in the treatment and control of T2D and associated diseases, still there is a lack of studies that have carried out a review on the acute effects of physical exercise on metabolic and hemodynamic markers and possible control mechanisms of these indicators in individuals with type 2 diabetes, not to mention that in a related way, these themes have been very little studied today. Mainly concerning the magnitude of different intensities and durations of exercise on glucose uptake, oxidation of macronutrients and blood pressure response after performing only one session of exercise (acute exercise) and biomolecular mechanisms involved in this phenomenon[1]. Hence, the aim of this study was to synthetize the current knowledge pertaining the acute effects of physical exercises in T2D; analyze the implications of exercise and determinate trends to future researches about this topic.

The method used in the present study was a review of the literature. As inclusion criteria and search of scientific articles, the following keywords were used: diabetes and exercise; diabetes and postexercise hypotension; diabetes and excess postexercise oxygen consumption; diabetes and acute effect of physical exercise, in the databases PubMed, Scielo and HIGHWIRE. The studies that have not addressed the acute effects of physical exercise on type 2 diabetes and did not show relevant results on the subject were excluded from the analysis.

ACUTE EFFECTS OF PHYSICAL EXERCISE ON GLYCEMIA AND INSULINEMIA

The control of glycemia is dependent of the activities of the neuroendocrine system. In resting conditions, the glucose uptake by the cells is mainly insulin dependent, where the glucose transporter 4 (GLUT-4) is translocated to the cell membrane, facilitating glucose entrance in the cell cytoplasm[10]. During exercise, an increase in the uptake and utilization of glucose occurs, and it seems to be dependent on the intensity and duration of the effort. The more intense the effort is, more carbohydrate will be metabolized[11,12]. Therewith, exercise promotes a reduction in glycemia, which is initially controlled by glucagon, epinephrine and norepinephrine. Afterwards, with the assistance of growth hormone and glucagon, production and release of glucose by the liver in the bloodstream is increased, thus, regulating again the glycemia[13].

This acute effect of exercise is benefic in euglycemic and T2D individuals. Exercise increases the concentration of GLUT-4 in the cell membrane, which leads to the increase in glucose uptake, even with low insulin levels[14]. On the other hand, the mechanisms surrounding this phenomenon are still inconclusive. Higher expression of key-proteins related to the insulin pathway, such as insulin receptor substrate 1 and phosphatidylinositide 3-kinases, and insulin independent mechanisms, such as the increase in the activity of AMP-activated protein kinase, the activation of the calcium-calmodulim pathway, and the kallikreins-kinins components can be involved in this process[10,15-20].

Furthermore, both exercise models, aerobic and resistance, promote improvements in glucose tolerance, insulin sensitivity and reduction in glycemia between 2-72 h, which seems to be dependent on the intensity and duration of the effort[1,21,22].

Although, there is some knowledge about the benefits of acute exercise in T2D, more studies are still made necessary to elucidate some questions, such as the effects of intense exercise in general population, since the most studies and exercise prescription to this population are of moderate intensity[1].

CARBOHYDRATE AND FAT OXIDATION DURING AND POST EXERCISE IN T2D

Insulin resistance, along with elevated oxidative stress, impairs energy metabolism at rest, as well as during and after exercising in T2D. At rest, the lowest availability of glucose and muscular glycogen in T2D increases the predominance of fat oxidation when compared to euglycemic individuals[1].

Although glucose uptake by insulin dependent pathways are impaired in T2D, exercise increases carbohydrate

oxidation, and this capacity seems to be preserved in T2D, since the glucose uptake during the effort occurs mainly by insulin independent pathways[23]. Nevertheless, T2D demonstrates lower capacity to utilize carbohydrate during exercise when compared to euglycemic individuals[24].

Other peculiarities occur during exercise in T2D, such as the decrease in rate of fatty acids oxidation when compared to euglycemic[25]. However, the effects of different lactate threshold intensities, during and after aerobic exercises, have been little studied and are yet inconclusive.

Ghanassia *et al*[25] observed that the predominance of carbohydrate oxidation in T2D during exercise seems to be independent of the intensity of effort. Nevertheless, the use of carbohydrate as substrate seems to be dependent of intensity, since it is available in the muscle (glycogen) and in the blood (glucose)[1].

Lima *et al*[26] observed an increase in fat oxidation after a cycle ergometer session, when compared to resting values in T2D. Furthermore, high exercise intensities extend this increase, and fat oxidation after exercise was higher in T2D in comparison to euglycemic.

The increase in carbohydrate oxidation during exercise, as well as fat oxidation during the post exercise recovery period can contribute to augment insulin sensitivity, and collaborate to reduce body fat percentage. It is noteworthy that the accumulation of intramuscular fat has a direct relation on insulin resistance, and consequently the appearance of T2D[27,28].

POST-EXERCISE HYPOTENSION IN T2D

Individuals with T2D present other impairments, such as endothelial dysfunction[3,29], increased sympathetic tonus and other cardiovascular diseases, including hypertension[30], which lead to the increase in morbidity and mortality.

One session of aerobic or resistance exercise can promote postexercise hypotension (PEH). The exercise-induced mechanical stress on the wall of the arteries, can increase the release of vasodilating substances by the endothelium (*e.g.*, nitric oxide, bradykinin), augments baroreflex sensitivity, and decreased sympathetic nervous activity in the solitary tract nucleus caused by the release of substance P by skeletal muscles[31-34]. This adaptation can bring benefits to health, because it helps to keep low levels of blood pressure, avoiding and controlling blood pressure increase at rest. However, the magnitude of this effect seems to be diminished in T2D individuals, since this population presents endothelial dysfunction, which collaborates to a decrease in the capacity of nitric oxide (NO) release when compared with euglycemic individuals[35-37]. Increased sympathetic tonus and other cardiovascular diseases are also observed in T2D[30].

Studies have demonstrated that the occurrence of PEH in T2D can be intense depending on the effort. Lima *et al*[4] demonstrated that T2D individuals seem to be more responsive to high intensity exercise sessions, since exercise above lactate threshold (LT) (110% of the LT) resulted in a significant decrease in systolic blood pressure (SBP) values up to 90 min after the session, whereas exercise performed below lactate threshold (90% of the LT) only reduced SBP during 45 min post exercise.

Simões *et al*[38] comparing two resistance training exercise intensities (23% and 43% of 1RM), observed that only the higher session (43%) promoted PEH. Similar results were found by Motta *et al*[29], when studying the effects of a 20 min high intensity cycle ergometer (90% of lactate threshold) in individuals with and without T2D. Both studies only observed significant blood pressure decreases in non T2D individuals.

Although the physiological mechanism responsible by this process still remains inconclusive, it is known that high intensity exercise promotes increases the activity of the kallikrein-kinin system, and consequently, augments the synthesis and release of nitric oxide[29]. However, more studies are still made necessary to elucidate this question.

EXCESS POSTEXERCISE OXYGEN CONSUMPTION IN T2D

Exercise increases oxygen consumption after exercising and during rest, this phenomenon is known as excess postexercise oxygen consumption (EPOC), which has a fast component (2-3 min), and a slow component which can persist for more than 30 min. The duration and magnitude of EPOC depends on the intensity and duration of the effort[39-42].

The need to resynthesize creatine phosphate, restore intramuscular oxygen, body temperature and muscular glycogen, increased activity of the sodium-potassium pump, clearance of lactate, high levels of epinephrine and norepinephrine are factors that can lead to EPOC[40,41].

However, T2D individuals present metabolic impairments, such as lower capacity to utilize carbohydrate, due to lower enzymatic regulation and intracellular signalling and gene transcription[43]. Thus, these modifications can change the pattern of metabolic and respiratory alterations elicited during and after exercise[4]. Therefore, it decreased the benefits of EPOC when compared to euglycemic individuals.

Studies about EPOC in T2D are scarce. Therefore, determining which intensity and duration could be more beneficial to promote this event in T2D is important to increase post-exercise fat oxidation, once the accumulation of intramuscular fat has been associated to the development of T2D[43].

NITRIC OXIDE AND EXERCISE IN TYPE 2 DIABETES

NO is a gaseous, inorganic and colorless free radical, which has seven electrons of nitrogen and eight of oxygen, having an unpaired electron[44]. NO is synthetized from oxidation one of the two guanidine nitrogens of

L-arginine, which is converted to L-citrulline[45].

NO produced by endothelial cells has an essential function in the process of relaxing of blood vessels. In physiological conditions, vascular relaxing occurs when the membrane receptors of endothelial cells are activated by soluble stimulus, which include: acetylcholine, bradykinin, adenosine diphosphate, substance P, serotonin and others, or when there is an increase of friction exerted by circulating cells in the endothelial layer (shear stress), generating the activation of endothelial NO synthases (eNOS) present in these cells, causing an increase of synthesis and release of NO[46].

NO produced by eNOS in endothelial cells spreads out to smooth muscle cells and vascular lumen. In the smooth muscle, NO interacts with the iron from heme group of enzyme guanylate cyclase (GC), causing an alteration in the structure of this enzyme, becoming activated (GCa). GCa catalyzes the departure of two phosphate groups from the molecule guanosine triphosphate, similar to the adenosine triphosphate (ATP), forming the cyclic guanosine monophosphate (cGMP). An increase in the levels of cGMP occurs when NO activates GC inside the cells[47], resulting in: (1) maintenance of vascular tonus; (2) blood pressure regulation; (3) prevention of platelet aggregation (by increase of cGMP and decrease in Ca^{2+}); (4) inhibition in adhesion of monocytes and neutrophils in the vascular endothelium; (5) anti-proliferative effect; and (6) anti-oxidant effect decreasing the production of peroxynitrite anion (ONOO-)[36]. Recent studies have shown that having a physically active lifestyle can contribute to maintain the functional capacity of the vascular endothelium, measured by the preservation of ability to produce NO[48-50].

The acute effects of exercise in the bioavailability of NO in physical performance and health, mainly in endothelial function, have been previously studied. Studies have demonstrated that exercise promotes an increase in NO levels after a single session. This acute effect of exercise in NO can induce positive adaptations in the cardiovascular, hepatic, esqueleto muscle systems and others[35,51].

This effect can influence health parameters, such as the control of blood pressure (BP). Faria *et al*[52] induced spontaneously hypertensive rats to one session of exercise (squat), using vests as load. They observed a decrease in BP, lower vascular reactivity, and endothelium-dependent vasodilatation mediated by the NO after exercising.

Augeri *et al*[53] examined the influence of the T786C gene of eNOS in post-exercise hypotension (PEH) and NO after a low (40% VO_2max) and moderate intensity exercise (60% VO_2max) in the cycle ergometer in prehypertensive individuals. The individuals, who carried the TT genotype, demonstrated less PEH than heterozygous individuals 9 h after exercising.

On the other hand, Long *et al*[54] determined the preventive effects of exercise in the coronary blood flow and macrovascular atherosclerosis in aerobic trained Yucatan pigs, which passed by a high cholesterol and fat concentrated diet. The short aerobic training kept the endothelium independent relaxation (adenosine) and increased the coronary endothelium-dependent relaxation through the action of bradykinin, that is a mediator of NO production, and decreased the developing of atheromatous plaques in the aerobic trained pigs.

In the venous system, Chies *et al*[55] evaluated the effects of angiotensin Ⅱ in the portal vein and vena cava of trained rats. The exposition of trained animals to consecutive sessions of acute aerobic exercise in a treadmill improved the portal vein response in the presence of angiotensin Ⅱ. This upgrading seems to be specific in portal vein, once the researches didn't observe this phenomenon in vena cava. The authors concluded that these adaptations are influenced by NO, endothelin and prostanoids.

Regarding vascular damage, Cubbon *et al*[56] studied the association of NO induced by exercise in the proliferation and mobilization of circulating progenitor cells (CPC), which are potential mediators of cell repair. The mobilization of CPC is critically dependent of NO, and south Asians are associated with low CPC levels. The mobilization of CPC was measured during a moderate-intensity exercise session. Mediators of vasodilatation and CPC were lower in the Asian group than in Europeans. During the exercise, the CPC also was lower in Asians. A decrease in the release of NO can contribute to inappropriate balance between vascular damage and muscular repair in the population.

The acute effects of exercise in NO have also been studied in other tissues. In the skeletal muscle, Lee-Young *et al*[57] observed that in mice without eNOS, ATP is reduced (40%), in sedentary conditions exercise tolerance is markedly impaired during a 30 min session. The researchers observed that a partial reduction of eNOS expression is enough to induce physiological changes in ATP and NO production, and consequently, reducing the tolerance to the effort.

Besides exercise, diet also seems to influence the availability of NO. Bailey *et al*[58] administrated oral L-arginine in nine healthy individuals and performed "step" exercise in two intensities (moderate and high) one hour after ingestion. Plasma nitrite was significantly higher in the group that consumed L-arginine, resulting in a decrease in SBP. Submaximal VO_2max was 7% lower in the moderate intensity exercise, while in the high intensity exercise the slow component was reduced and the time to exhaustion delayed with L-arginine supplementation. As a conclusion, the authors stated that diet with L-arginine showed similar results with nitrite, increasing the bioavailability of NO, and reducing the cost of O_2 in the moderate exercise and time to exhaustion in the maximal exercise.

One exercise session seems to increase the bioavailability of NO, collaborating with the regulation of vascular tonus, balance between damage and muscle repair and preventing diseases such as atherosclerosis and hypertension[59]. Studies related to the bioavailability of NO in different exercise intensities are inexistent. The production

Table 1 Summary of human studies about acute effects of physical exercise in type 2 diabetes

Ref.	Sample	Exercise intervention	Results
Lima *et al*[4]	T2D = 11	20 min of cycle ergometer at 90% and 110% LT, and control session	Higher intensity exercise (110% LT) was more effective than lower intensity (90% LT)
Sriwijitkamol *et al*[5]	Obese T2D = 12 Obese CG = 8 Lean CG = 8	40 min of cycle ergometer at 50% and 70% VO_{2max}	Obese and T2D had attenuated exercise-stimulated AMPK activity and AS160 phosphorylation. T2D had reduced basal *PGC-1* gene expression but normal exercise-induced increases in PGC-1 expression
Borghouts *et al*[12]	T2D = 8 CG = 8	1 h of cycle ergometer at 40% VO_{2peak} and control session	Muscle glycogen oxidation was lower in T2D than in CG. Plasma glucose contributed more to energy expenditure in T2D than CG
Braun *et al*[24]	Insulin-resistant = 6 Insulin-sensitive = 6	50 min of treadmill walking at 45% VO_{2max}	Carbohydrate oxidation and estimated muscle glycogen use were significantly lower in the insulin-resistance group
Ghanassia *et al*[25]	T2D = 30 CG = 38	Increasing exercise intensity in cycle ergometer	Lipid oxidation was lower in T2D. Maximal lipid oxidation point and the crossover point were lower in T2D
Lima *et al*[26]	T2D = 9 CG = 11	20 min of cycle ergometer at 90% LT, increasing exercise intensity and control session	T2D have a better fat oxidation after high-intensity exercise than moderate exercise. T2D had less fat oxidation than CG after moderate exercise
Motta *et al*[29]	T2D = 10 CG = 10	20 min of cycle ergometer at 90% LT and control session	CG presented PEH, but not in the T2D. Plasma kallikrein activity increased postexercise in the CG, but not in the T2D
Simões *et al*[38]	T2D = 10 CG = 10	Resistance exercise circuit at 43% and 23% 1RM (approximately 25 min), and control session	43% 1RM promoted PEH, whereas the 23% did not
Asano *et al*[60]	T2D = 11	20 min of cycle ergometer at 80% and 120% LT, and control session	Exercise above LT (120% LT) increase nitric oxide and decrease SBP post-exercise, but about 80% LT not

T2D: Type 2 diabetics; LT: Lactate threshold; CG: Control group; VO_{2max}: Maximal oxygen uptake; VO_{2peak}: Peak oxygen uptake; PEH: Post-exercise hypotension; 1RM: 1-repetition maximum; AMPK: AMP-activated protein kinase; PGC-1: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

of knowledge about this important topic is essential to define better exercise strategies to increase the bioavailability of NO in individuals with T2D after one exercise session.

A summary of acute effects of physical exercise in T2D, along with the reference, number of volunteers and the kind of intervention, can be observed in Table 1.

CONCLUSION

A single session of exercise can promote beneficial effects regarding blood pressure control, glycemia, carbohydrate oxidation during exercise and fat oxidation after exercise. Evidence has shown that exercise, especially at intense domains, can increase the bioavailability of nitric oxide, promoting a decrease in blood pressure after exercising. Furthermore, metabolic stress from exercising is able to increase the oxidation of carbohydrates during exercise, keeping an elevated O_2 consumption after exercising. This, in consequence, increases fat oxidation during at rest and improves glucose tolerance, insulin sensibility and can reduce glucose levels between 2 to 72 h depending of intensity and duration of the effort.

These acute effects of physical exercise are important to T2D, because they help to improve conditions such as high blood pressure, hyperglycaemia and lipidemia.

ACKNOWLEDGMENTS

The authors are grateful for the students' scholarships at undergraduate (CNPq), masters (CAPES) and PhD (CAPES and CNPq), as well as for the research productivity scholarships (CNPq).

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P- Reviewer: Pamidi N, Ray S **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (4): Diabetes-related complications

Is the present cut-point to define type 2 diabetes appropriate in Latin-Americans?

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Received: August 25, 2014 Revised: October 25, 2014
Accepted: November 17, 2014
Published online: December 15, 2014

Abstract

The diagnosis of diabetes mellitus type 2 (DM2) is based either on increased plasma glucose or Glycated hemoglobin levels. Since these measures are the only means for diagnosis of DM2, they must be well adapted to each population according to their metabolic characteristics, given that these may vary in each population. The World Health Organization (WHO) determined the cut-points of plasma glucose levels for the diagnosis of DM2 by associating hyperglycemia with the risk of a specific microvascular complication-retinopathy. Cardiovascular diseases are however the principal causes of mortality in patients with DM2 and we reported that in the Colombo-Ecuadorian population impaired fasting glucose and impaired glucose tolerance are both risk markers for myocardial infarction. We propose that the current cut-points accepted by the WHO need to be revaluated in populations such as Latin America and that there should be lower cut points for glycaemia in this population, to reduce the prevalence of cardiovascular complications associated with DM2.

Key words: Type 2 diabetes; Cut-off points; Cardiovascular diseases; Plasma glucose; Coronary disease

Core tip: We propose that the current cut-points to define type 2 diabetes accepted by the World Health Organization need to be revaluated in populations such as the Latin America and that there should be lower cut points for glycaemia in this population, to reduce the prevalence of cardiovascular complications associated with diabetes mellitus type 2.

Original sources: López-Jaramillo P, Velandia-Carrillo C, Gómez-Arbeláez D, Aldana-Campos M. Is the present cut-point to define type 2 diabetes appropriate in Latin-Americans? *World J Diabetes* 2014; 5(6): 747-755 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/747.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.747

INTRODUCTION

The World Health Organization (WHO) issued technical reports relating to diabetes in the years 1965[1], 1980[2], 1985[3], and 1999[4]. Over this period, there have been significant changes in the diagnostic criteria and for the classification of diabetes mellitus (DM) and intermediate hyperglycemia[5], also known as dysglycemia or prediabetes. In the first report in 1965, the WHO set a DM cut-off of $\geqslant$ 130 mg/dL according to the patient's response to a two hour oral glucose tolerance test (OGTT) and

their clinical manifestations[1]. Then in 1980, specific criteria were introduced, such as retinopathy or the presence of glucose in urine, or a random plasma glucose tests of ⩾ 200 mg/dL, and values for Fasting Plasma Glucose (FPG) of ⩾ 145 mg/dL or glucose in venous plasma 2-h after glucose load (75 g) ⩾ 200 mg/dL for the diagnosis of DM[2]. In 1985, the cut-off points for FPG were decreased to ⩾ 140 mg/dL while the OGTT of ⩾ 200 mg/dL was maintained[3].

In 1997, The Expert Committee of the American Diabetes Association (ADA) released their new recommendations for the classification and diagnosis of diabetes. The stage impaired glucose tolerance (IGT) was retained but there were several major changes including: (1) the preferred use of the terms "type 1" and "type 2" instead of "insulin-dependent" and "non-insulin-dependent" to designate the two major types of DM; (2) The analogous intermediate stage of fasting glucose was named "impaired fasting glucose (IFG)"; and (3) a lower cutoff for FPG from ⩾ 140 mg/dL to ⩾ 126 mg/dL to diagnose diabetes was established (this level of FPG having been found equivalent to the 200 mg/dL value in the oral glucose tolerance diagnostic test)[5].

In 1999, the WHO then amended the cut-off points to ⩾ 126 mg/dL in fasting glucose and maintained the ⩾ 200 mg/dL for OGTT, which was established in 1980. The new fasting criterion was chosen to represent a value at the upper end of the range, which in many patients corresponds to the diagnostic significance of the 2-h post-load concentration, which was not modified[4].

The criteria currently used for the diagnosis of diabetes and intermediate hyperglycemia have been in place globally for almost a decade, and are widely accepted by the ADA[6] and the WHO[7,8] using the four following criteria: Symptoms of hyperglycemia such as polyuria, polydipsia, and unexplained weight loss, and a casual plasma glucose ⩾ 200 mg/dL; casual-defined as a result obtained at any time of the day; (2) A 2-h plasma glucose ⩾ 200 mg/dL during an OGTT. This test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water; (3) Fasting glycemia levels ⩾ 126 mg/dL; and (4) Glycated Hemoglobin (HbA1c) ⩾ 6.5%. Both the ADA and the WHO believe that sufficiently stringent quality assurance tests are in place and that assays are standardized to criteria aligned to the international reference values, so that there are no conditions present which preclude an accurate measurement of HbA1c.

HOW WERE THE CUT-OFF POINTS FOR DM DETERMINED?

While plasma glucose and HbA1c represent the basic criterion measures to define DM, the universally utility of these determinations has been questioned[9]. The diagnostic cut-off points for diabetes were based on two sets of evidence: (1) Plasma glucose levels associated with an increased risk of specific microvascular complications, particularly retinopathy; and (2) The distribution of plasma glucose in the general population[9-11].

However, there are a number of methodological weaknesses of the studies that have reported the cut-points for increased risk of retinopathy including inadequate statistical power for this type of analysis[10]. Moreover, these studies used different methods to diagnose retinopathy and some used patients already identified as diabetic, while others used non-diabetic patients[10,11]. In addition, some reports included people with diagnosed DM who were receiving blood glucose lowering treatment introducing a bias associated with treatment-induced effects on plasma glucose. Excluding people with treated diabetes from analyses eliminates the bias related to the treatment effect, but changes the characteristics of the diabetic population[12].

One of the most important studies to support the cut-points was conducted by Ito *et al*[11], which included 12.208 people and began in 1965 and lasted until 1997. The authors reported a significantly increased prevalence of retinopathy at a baseline FPG cut-point of 125 mg/dL and 198 mg/dL in 2-h post-glucose load.

Other microvascular complications are more weakly associated with plasma glucose levels than retinopathy[13]. Studies which have examined the relationship between plasma glucose and proteinuria, reported a significant association but weaker than with retinopathy[13]. For instance, among patients with DM, only 20%-40% of patients with microalbuminuria will progress to overt nephropathy, and only 20% will go on to end-stage renal disease within the next 20 years[14]. Moreover, the data showing a relationship between plasma glucose and biopsy confirmed diabetic renal disease is not totally convincing, since the prevalence of non-diabetic nephropathy in the patients with DM who underwent renal biopsy varies from 10% to 85% in different reports[15]. Furthermore, FPG and HbA1c values associated with the presence of diabetic nephropathy were exceptionally high: 183 ± 61.9 mg/dL and 8.6% ± 2.4%, respectively[16].

The distribution of plasma glucose in the general population was another source of data used to define cut-points. In 2006, the WHO reported that the distribution of plasma glucose among the population was either unimodal, in which the entire population is represented by a single curve, or bimodal, represented by two overlapping curves[7]. However, an analysis of DETECT-2, representing plasma glucose data measured during an OGTT in 26 different countries, found a wide variation in cut-points[9]. Cut-points for FPG in different countries ranged from 103 to 153 mg/dL (median 128.5 mg/dL), and for 2-h plasma glucose from 164.7 to 323.9 mg/dL (median 224.4 mg/dL). Moreover, when known diabetes was removed from the analysis, the distributions of plasma glucose do not generally give rise to a bimodal structure that is useful for deriving a cut point for diabetes. Thus, bimodality seems not to be a suitable method for defining diagnostic cut points for diabetes in population studies which include people of different origin[9].

Bimodal distribution has also been reported in a

number of populations with a high prevalence of diabetes, including the American Pima Indian, Micronesian of Nauru, Egyptian, Mexican, Papua New Guinea, and South African populations[9,17]; while few studies on bimodality have been conducted in populations with a low prevalence of diabetes[18].

Recently, and in support of the use of HbA1c as a diagnostic criterion, several studies have noted that HbA1c reflects average plasma glucose and does not require any special preparation such as fasting. These features led to it becoming the gold standard for assessing glycemic control in people with diabetes, and it has also become a means to assess glucose tolerance in those with undiagnosed diabetes[12]. The relationship between HbA1c and the presence of retinopathy is similar to that of plasma glucose, making it at least as accurate in defining the level of hyperglycemia at which retinopathy prevalence increases[19].

Moreover, HbA1c has appreciable superior technical attributes, including less pre analytic instability and biological variability, and is a more clinically convenient measure. HbA1c has been demonstrated to be more reliable than FPG, with a day to day coefficient of variation of less than 2% compared to 16% for FPG[20].

Studies have now established an HbA1c level associated with an increase in the prevalence of moderate retinopathy, providing strong justification for assigning an HbA1c cut-off point of $\geqslant$ 6.5% for the diagnosis of diabetes[8]. Although this cut-off point must not be used as an absolute dividing line between normal glycemia and diabetes, this value is sufficiently sensitive and specific to identify individuals who are at risk of developing retinopathy and who therefore, should be diagnosed as diabetic[20].

HbA1c however does have some limitations which should be considered when using it as criteria for the diagnoses of DM. First, the cost of the test precludes its routine use. Second, there are some specific conditions that can influence and therefore preclude HbA1c testing, including the following hemoglobin traits: HbS, HbC, HbF, and HbE, as well as various types of anemias, pregnancy, uremia and blood transfusions[21]. Some of these factors may represent an additional problem in under-resourced countries, due to their higher prevalence of anemia and hemoglobinopathies[21]. Moreover, it should be noted that there are normal age-related increases in HbA1c[22].

PROPOSED MECHANISMS TO EXPLAIN THE NEGATIVE EFFECTS OF HYPERGLYCEMIA ON THE VASCULAR WALL

Blood glucose level can also be a risk marker for cardiovascular diseases (CVD) among apparently healthy non-diabetic individuals[23-26]. The effects of elevated glycemia levels include non-enzymatic glycosylation of proteins, increased metabolism of glucose through the polyol and glucosamine pathways and the generation of free radicals[27-32]. Glycosylation of low-density lipoprotein makes it more susceptible to oxidization and therefore more atherogenic[27]. Advanced glycosylation end products (AGEs) can cross-link proteins, particularly in the extracellular matrix of the vascular wall[31,32]. Metabolism of excess glucose by secondary pathways can also alter cell function by modifying signal transduction and changing the oxidative potential of cells[30]. This may contribute to general cell damage and dysfunction[28]. These pathways can also activate tissue-specific protein kinase C[29] and increase in the activity of which decreases fibrinolysis and nitric oxide (NO) levels and increases cell proliferation and coagulation, contributing to the progression of CVD[28-30].

The association between intermediate hyperglycemia and coronary heart disease has been explained by the predisposition of these subjects to subsequently present DM2, a condition that as noted above, is directly related to the development of CVD[27]. However, hyperglycemia *per se* may also be directly involved in the development of atherosclerosis by promoting metabolic and structural changes in the endothelium that eventually produce irreversible damage. Therefore, the association between hyperglycemia and cardiovascular risk should be considered as a continuum, rather than one that depends only on reaching a specific cut point.

Experimental studies suggest that hyperglycemia reduces the activity of NO at the vascular endothelial level[28]. Hyperglycemia induces a series of cellular events that increase the production of reactive oxygen species that inactivate NO and lead to the formation of peroxynitrite[29,30]. In addition, mitochondrial production of reactive oxygen species increases the intracellular formation of AGEs[30], which affect endothelial function and activate the receptors for AGEs causing apoptosis and altered vascular structure[31-33]. In non-diabetic subjects, altered levels of post-load glucose have been associated with the presence of structural alterations at the level of the carotid arteries, manifested by increased carotid intima-media thickness[34-36]. Moreover, chronic hyperglycemia can also cause cellular structural changes, which would explain the known point of no return for the micro and macrovascular complications observed in diabetic patients[37-39]. Recent experimental studies with rats in which diabetes was induced using streptozotocin, demonstrated a loss of nitric oxide synthase function (NOS) in nitrergic neurons. This effect was mediated by an increased production of AGEs, oxidative stress and neuronal apoptosis, which was reversible only when treatment with insulin was introduced in early stages. After 12 wk of streptozotocin-induced diabetes, insulin therapy was not able to recover the function of the nitrergic neurons, which had suffered an increased apoptosis[37,38]. These experiments suggest that chronic hyperglycemia over time leads not only to an alteration of NOS function, but also in later stages to irreversible structural changes in different tissues. Since streptozotocin-induced DM is more similar to type 1 DM, it is therefore possible that the

underlying mechanism of vascular damage in type 2 DM is different to that described above. Nonetheless, this mechanism could be responsible for the development of atherosclerosis in the vascular wall of hyperglycemic patients. Thus, it is attractive to postulate that in the early stages of hyperglycemia, the use of hypoglycemic treatments could decrease the formation of AGEs, reversing endothelial dysfunction and preventing both structural disorder and the progression to CVD[39].

WHY SHOULD CUT POINTS OF PLASMA GLUCOSE TO DIAGNOSE DIABETES MELLITUS BE RE-EVALUATED?

We propose that CVD prevention depends on an early and aggressive intervention to control glycemia levels, probably at the prediabetes stage, to avoid reaching a "point of no return" with respect to structural alterations of the arterial walls. This proposal is supported by important clinical trials[40-44] such as the United Kingdom Prospective Diabetes Study which demonstrated that if an intensive treatment of hyperglycemia is started when DM2 is first diagnosed, there is a significant decrease in the number of cardiovascular events[41], maintained until 10 years after end of the study[40]. However, as recently demonstrated in clinical trials, if the intensive treatment is started after 8[42], 10[43], or 12[44] years of diagnosed DM2 the impact of the intensive treatment does not produce a decrease in the number of cardiovascular events (Table 1). These results highlight the importance of starting the hypoglycemic intervention earlier than is common practice currently.

The magnitude of the glycemia association with CVD risk has been reported in many studies[25,45], and although post-load blood glucose level has a linear relationship with CVD risk in the non-diabetic range, a possible threshold effect for FPG level appears to exist around 100 mg/dL[27]. There is an important body of information indicating that the cardiovascular risk starts at levels well below the cutoff point currently used for the diagnosis of DM2 and increases continuously[25,46]. Many studies show that non-diabetic patients with hyperglycemia have an increased risk of cardiovascular morbidity and mortality[46-51]. The meta-analysis of prospective studies conducted by Levitan *et al*[23] shows that the group with the highest post-load blood glucose level (midpoint range, 150-194 mg/dL) had a 27% greater relative risk (RR) for CVD compared with the group with the lowest level (midpoint range, 69-107 mg/dL) (RR = 1.27, 95%CI: 1.09-1.48).

Moreover, in a meta-analysis of studies that included a total of 95.783 people, Coutinho *et al*[25] found a linear relationship between glucose levels and subsequent cardiovascular events over a period of 12 years, reporting a RR = 1.33 (95%CI: 1.06-1.67) for those with FPG levels of 110 mg/dL and an RR of 1.58 (95%CI: 1.19-2.10) for patients with post-load blood glucose levels > 140 mg/dL.

The Whitehall Study[51] lasted 33 years and followed 17.869 male civil servants aged 40-64 years, of which 3.561 died of coronary diseases. In this study, the hazard of coronary mortality rose when 2-h blood glucose level reached 83 mg/dL (95%CI: 76-96). Between this level and 200 mg/dL, the age-adjusted hazard ratio was 3.62 (95%CI: 2.3-5.6). Although the data was applied at baseline in these male civil servants, this report has a limitation in that the findings are based on a 50 g OGTT, and a slightly differing dose-response relationship might be obtained with a 75 g glucose load.

The DECODE study[45] was a prospective European analysis of 22 cohorts with baseline glucose measurements for 29.714 subjects aged 30-89 who were followed-up for 11 years. After adjusting for other cardiovascular risk factors, the study reported an association between risk of death and both high glucose concentrations and very low glucose levels. Compared with a fasting plasma glucose of 81-110 mg/dL, the multivariate adjusted HR (95%CI:) for FPG < 81 mg/dL was 1.2 (1.0-1.4) for all causes, 1.3 (1.0-1.8) for CVD, and 1.1 (0.9-1.4) for non-cardiovascular mortality. For 2-h plasma glucose of 54.4-81 mg/dL, as compared with 2-h plasma glucose of 81.6-100 mg/dL the HRs were 1.1 (1.0-1.2) for all causes mortality, 1.1 (0.9-1.3) for cardiovascular mortality, and 1.1 (1.0-1.3) for non-cardiovascular mortality, respectively.

In the Asian Pacific Region, blood glucose data from 237.468 participants of 17 cohort studies are available[52]. Continuous positive associations were demonstrated between usual fasting glucose and the risks of cardiovascular diseases down to at least 88.6 mg/dL. Overall, each 18 mg/dL lower than usual fasting glucose was associated with a 21% (95%CI: 18%-24%) lower risk of total stroke, and 23% (95%CI: 19%-27%) lower risk of total ischemic heart disease. The associations were similar in men and women, across age-groups, and in Asian compared with Australasian (Australia and New Zealand) populations.

The China Heart Survey[53], a multicenter study, recruited 3.513 patients hospitalized for Coronary Artery Diseases (CAD), of whom 35.1% were admitted for acute CAD and 64.9% were elective admissions for CAD. At entry, 1.153 patients (32.8%) had known DM and 97 (2.7%) had newly diagnosed DM. Furthermore, 32.6% had IGT, and 4.7% had IFG. The proportion of patients with diagnosed DM increased from 32.8% at baseline to 52.9% post-OGTT analysis.

The GAMI study[54] of 181 patients admitted to two Swedish hospitals with acute myocardial infarction (AMI) and no history of DM, found a prevalence of 34% for prediabetes and 33% for de novo DM, leaving only 33% with no alteration in glucose metabolism. This distribution was similar when measurements were repeated at 3 and 12 mo. These findings were later confirmed by another study that included 4.961 patients with coronary disease enrolled in 110 centers throughout Europe[55]. In this study the prevalence of pre diabetes was 32% in those patients admitted with acute coronary syndrome and only 29% of enrolled patients had a normal carbohydrate metabolism.

Table 1 Differences in cardiovascular outcomes according to the time of disease (diabetes mellitus type 2) before the start of an intensive hypoglycemic intervention

Study	Time since diagnosis	Treatment	Mean outcomes
UKPDS 34 and 80[40,41]	Newly diagnosed	Metformin added to an experimental group, median glycated hemoglobin was 7.4% in the metformin group compared with 8.0% in the conventional group	↓ 32% for any diabetes-related endpoint ↓ 42% for diabetes-related death ↓ 36% for all-cause mortality A continued reduction in microvascular risk and risk reductions for myocardial infarction and death from any cause were observed during 10 yr of post-trial follow-up
The Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation trial[42]	7.9 yr	Gliclazide (modified release) plus other drugs as required to achieve a glycated hemoglobin value of 6.5% or less and Perindopril + Indapamide	No significant effects on major macrovascular events, death from cardiovascular causes, or death from any cause
The Action to Control Cardiovascular Risk in Diabetes trial[43]	10 yr	Individualized intensive therapy of a combination of any hypoglycemic drug targeting a glycated hemoglobin level below 6.0% or standard therapy targeting a level of 7% to 7.9%	The intensive-therapy group did not differ significantly from the standard-therapy group in the rate of the primary outcome (a composite of nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes) but had more deaths from any cause (primarily cardiovascular)
The Veterans Affairs Diabetes Trial[44]	11.5 yr	Intensive-therapy group goal was an absolute reduction of 1.5% in the glycated hemoglobin level, as compared with the standard-therapy group, metformin plus Glimepiride or Rosiglitazone	No significant effect on the rates of major cardiovascular events, death, or microvascular complications

UKPDS: United Kingdom Prospective Diabetes Study.

In Latin America, the ongoing multicenter Colombian-Ecuadorian study which includes until now 439 subjects distributed in 8 hospitals of Colombia and Ecuador to determine the prevalence of pre diabetes in patients with a first AMI shows that the combined prevalence of DM2 and prediabetes is 69.47%. Ninety subjects (20.50%) presented with antecedents of DM2; another 85 (19.36%) were diagnosed with DM2 while hospitalized; and 130 (29.61%) presented with prediabetes. Only 134 subjects (30.53%) were normoglycemic[56].

The existence of a strong association between cardiovascular risk factors and IFG has also been reported in Colombia, with an even greater association with the presence of abnormal plasma glucose levels after an oral glucose load[57]. Additionally, in our population there is evidence indicating that hyperglycemia is common in patients with already established coronary disease[58].

Furthermore, a Colombian population study found that an IFG > 100 mg/dL was the risk factor with the highest degree of association with the presence of CAD in patients with stable angina pectoris, independent of the presence of other traditional cardiovascular risk factors[58]. Moreover, in this population fasting hyperinsulinemia and the socio-economic status of individuals with a first myocardial infarction were the only factors that remained significant predictors of a new cardiovascular event after a multivariate analysis[59]. We have previously shown that Colombian people present a higher vulnerability to present with insulin resistance at lower levels of abdominal obesity in youth adults[60,61], in pregnancy[62], and in children[63].

Many years ago Hales and Barker demonstrated that low birth weight is associated with an increased risk of developing obesity, metabolic syndrome and DM2[64-66]. Based on the results of their pioneering work and subsequent confirmatory studies, we have proposed[67-69] that the fetal programming during pregnancy of women that have deficient nutrition and/or an increased frequency of subclinical infection and preeclampsia, have an increased risk of giving birth to a low birth weight child with a higher risk of subsequently developing insulin resistance (IR) and low degree inflammation. It is well established that children with low birth weight have a decreased mass of beta cells, nephrons, hepatocytes, and fewer muscle fibres. We recently demonstrated, in children and adolescents that low muscle strength is associated with increased adiposity, C-reactive protein, HOMA index and metabolic risk factors, and that this association was stronger in with low birth weight[70]. Moreover, in a sub analysis of the ORIGIN study[71] we demonstrated that low handgrip strength is an important factor associated to an increased risk of cardiovascular mortality in prediabetic and diabetic patients[71]. To explain these results we have proposed that the dramatic increase of overweight and obesity, especially abdominal adiposity, in low and medium income countries[72], is promoting epigenetic adaptations which may alter the leptin/adiponectin (L/A) ratio. This L/A disturbance is in turn the determinant, in populations of low and medium income countries, of their increased vulnerability to the development of IR and an increased risk of cardiovascular events at levels of glycemia that are lower than those used to define DM2[73-76]. Moreover, there are possible regional differences in the risk of developing IR, DM2 and CVD as-

sociated with prediabetes and DM2, as we have recently demonstrated in relation to lung function[77].

PERSPECTIVES TO MODIFY THE CUT-OFF POINTS OF DM RELATED WITH THE RISK OF MACROVASCULAR COMPLICATIONS

The term diagnosis has typically been reserved to characterize or identify individuals with a specific disease. Because the term implies a condition that causes symptoms, tests are often required to confirm the diagnosis. In this order of ideas, when selecting the threshold glucose values, the National Diabetes Data Group[78] acknowledged that "there is no clear division between diabetics and non-diabetics in the FPG concentration or in their response to an oral glucose load" and consequently values were established for each method to identify diabetic patients based on retinopathy and the distribution of plasma glucose population.

Epidemiological studies[10-12] that included an Egyptian population, Pima Indians and the US National Health and Nutrition Examination Survey, all identified retinopathy using fundus photography or direct ophthalmoscopy and by measuring glycemia using FPG, 2-h post-glucose load, and HbA1c, demonstrated that glucose level is a continuous risk factor for retinopathy: the higher levels the higher risk.

Deriving cut points for normal glycemia level from distributions of FPG and 2-h post-glucose load might not be suitable to define cut points for DM because metabolic regulation could varies from population to population. It might be more relevant to base the diagnostic criteria on thresholds for diabetes-specific macrovascular complications, which are probably lower than those for microvascular complications such as retinopathy. Data from the DECODE study[45] which was carried out on behalf of the European Diabetes Epidemiology Group showed that the number of patients diagnosed with DM was one third higher for men and 44% higher for women when using 2-h post-glucose load measurement than when using the FPG, confirming that the 2-h post-glucose load criterion is more accurate than FPG criteria to identify DM. HbA1c is recommended and used in many countries to diagnose DM[12,20]. However the high prevalence of anemia and hemoglobinopathies in under-resourced countries such as ours, together with its high cost, limits its use and from our point of view should not be for now, recommended as a diagnostic test.

The data of the previously mentioned Latin American studies indicate the presence of macrovascular diseases at glycemia levels lower than the internationally established cut points for DM2. These data suggest that the present cut-off points accepted for our population might not be accurate and might have to be reconsidered. Recent studies have shown that the association between dysglycemia and CVD has a considerable increase at levels as low as 100 mg/dL[25,27,45], and therefore, we consider the re-defined cut-points to diagnose DM2 should be around this value. Nevertheless, it is noteworthy that these studies have not been designed for this specific purpose and have not been conducted in Latin America. Thus, as with the risk of microvascular complications, several limitations will be found if we try to re-define the cut-points for DM2 on this basis.

Moreover, as lowering the cut-off points will substantially increase the prevalence of DM2, several public health consequences should be considered before this adjustment. Certainly, diabetic patients require more health care, leading to greater use of resources. In this context, an increased prevalence of DM2 could cause an initial financial challenge of the health systems and household economies in Latin American countries[79]. Nevertheless, indirect economic costs and social consequences attributable to premature mortality and temporary and permanent disability generated as complications of DM should be also considered. Indeed, the direct annual cost associated with diabetes for the year 2000 in Latin America and the Caribbean was estimated as 10721 million US dollars; whereas, the total indirect cost was estimated at almost 54496 million US dollars (mortality, permanent disability and temporary disability accounted for 6%, 92% and 2% of this amount, respectively)[80]. These results suggest a long-term positive cost-effective ratio of an early intervention.

Furthermore, health systems in Latin American countries are based on a model of care with a biomedical curative approach[81], and this has not been favorable in controlling the epidemic of DM2. Thus, health systems should move from an approach of treating DM2 to one of preventing DM2 and its complications. In this way, various socio-medical models are currently being evaluated in Latin-America, such as the ongoing HOPE-4 study in Colombia, in which we are inviting community leaders and non-professional health care workers to form part of the health team to implement new strategies for the detection, prevention and control of non-communicable chronic diseases.

In conclusion, the present challenge for Latin American countries is to conduct population studies in accord with our specific socio-economic conditions, which will permit to establish the cut-point after which lifestyle and/or pharmaceutical interventions must be initiated with the objective of preventing macrovascular complications, associated with hyperglycemia. Further research to assess the economic, public health, and social perspectives is also warranted.

ACKNOWLEDGMENTS

The authors declare that they have no competing interests.

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P- Reviewer: Charoenphandhu N, Traub M
S- Editor: Song XX **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Risks of rapid decline renal function in patients with type 2 diabetes

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Received: July 2, 2014 Revised: July 26, 2014
Accepted: September 17, 2014
Published online: December 15, 2014

Abstract

Progressive rising population of diabetes and related nephropathy, namely, diabetic kidney disease and associated end stage renal disease has become a major global public health issue. Results of observational studies indicate that most diabetic kidney disease progresses over decades; however, certain diabetes patients display a rapid decline in renal function, which may lead to renal failure within months. Although the definition of rapid renal function decline remained speculative, in general, it is defined by the decrease of estimated glomerular filtration rate (eGFR) in absolute rate of loss or percent change. Based on the Kidney Disease: Improving Global Outcomes 2012 clinical practice guidelines, a rapid decline in renal function is defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year. It has been reported that potential factors contributing to a rapid decline in renal function include ethnic/genetic and demographic causes, smoking habits, increased glycated hemoglobin levels, obesity, albuminuria, anemia, low serum magnesium levels, high serum phosphate levels, vitamin D deficiency, elevated systolic blood pressure, pulse pressure, brachial-ankle pulse wave velocity values, retinopathy, and cardiac autonomic neuropathy. This article reviews current literatures in this area and provides insight on the early detection of diabetic subjects who are at risk of a rapid decline in renal function in order to develop a more aggressive approach to renal and cardiovascular protection.

Key words: Type 2 diabetes; Diabetic kidney disease; Rapid decline; Estimated glomerular filtration rate; Albuminuria

Core tip: The progression rate of diabetic kidney disease is highly variable, a rapid decline of renal function can lead to renal failure within months. Risk factors account for rapid decline renal function in patients with type 2 diabetes include ethnic/genetic and demographic factors, lifestyle and health behaviors, advanced albuminuria, poor glycemic control, dyslipidemia and some biochemical abnormalities. Diabetic patients with retinopathy or cardiac autonomic neuropathy are at increased risk of a rapid decline in estimated glomerular filtration rate. Early detection of high-risk groups with a more aggressive multifactorial approach to renal and cardiovascular protection is important.

Original sources: Sheen YJ, Sheu WHH. Risks of rapid decline renal function in patients with type 2 diabetes. *World J Diabetes* 2014; 5(6): 835-846 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/835.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.835

INTRODUCTION

Type 2 diabetes is one of the leading causes of chronic kidney disease (CKD) worldwide, and diabetic kidney disease has become a major global public health issue[1]. Early detection and intervention in diabetic kidney disease can help to slow renal function decline, prevent complications, and decrease cardiovascular events, thereby improving survival and quality of life in type 2 diabetics[2]. However, potential causes accounting for variation in diabetic kidney disease and its rate of progression are still largely unexplored. In most cases, disease progresses over decades; however, a rapid decline in renal function can lead to renal failure within months[3]. Thus, in type 2 diabetics, defining high-risk groups and preventing or retarding disease progression is an emerging challenge. This review targets the potential risk factors of a rapid decline in renal function in patients with type 2 diabetes.

EPIDEMIOLOGY OF DIABETIC KIDNEY DISEASE

Diabetic kidney disease is identified clinically through the presence of albuminuria, impaired glomerular filtration rate (GFR), or both[4], and these two biomarkers have been used for the diagnosis, severity classification, and outcome prediction of CKD[5-8]. The categories of albuminuria are defined as microalbuminuria or macroalbuminuria based on a urinary albumin-to-creatinine ratio (UACR) of 30-300 mg/g, or > 300 mg/g, respectively[9,10], and impaired renal function is defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2[1,4,10]. International consensus on the incidence of CKD in patients with type 2 diabetes is lacking[11]. Although the prevalence of diabetic CKD is increasing worldwide, there are large differences between regions and ethnicities (Table 1). A report from the UK Prospective Diabetes Study (UKPDS), states that 1544 (38%) of 4031 patients developed albuminuria (microalbuminuria or macroalbuminuria), and 1449 (29%) of 5,032 patients developed renal impairment (based on the Cockroft-Gault formula of eGFR < 60 mL/min per 1.73 m^2) over a 15-year period[12]. Meanwhile, the Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes (DEMAND) study, in which data from 32208 type 2 diabetics from 33 countries were collected, reported that overall global prevalence of microalbuminuria and macroalbuminuria was 39% and 10% respectively, while eGFR below 60 mL/min per 1.73 m^2 occurred in 22% of the 11573 patients with available data[13]. According to the US Renal Data System (USRDS) 2013 report, 3 out of 5 new end stage renal disease (ESRD) patients came from diabetes in Malaysia, Mexico, and Singapore; furthermore in the United States, the odds ratios of diabetes in albuminuria (UACR more than 30 mg/g) and CKD (defined as eGFR below 60 mL/min per 1.73 m^2) were 3.9 and 2.1 respectively[14]. It was recently reported that 30% of CKD in 5584 Chinese patients aged 20-79 years, was associated with dysglycemia (diabetes and prediabetes), independent of age, sex, and hypertension status[15]. It should be noted that some limitations and pitfalls were identified in these epidemiological data, for example, demographic distribution[11], socioeconomic status[16], dynamic changes in the incidence of diabetes, changes in the use of medication (including anti-diabetic drugs and anti-hypertensive drugs), and the improvement of survival rates in diabetic and ESRD patients[11].

DEFINING A RAPID DECLINE IN RENAL FUNCTION

Annual decline in GFR in an individual varies widely depending on race, age, the presence of underlying conditions, the etiology of CKD, and the presence of comorbidities. A previous study reported that age-related eGFR decline is about 0.75-1 mL/min per 1.73 m^2 per year over 40 years of age[17]. Among the healthy population, eGFR decline is approximately 0.36-1.21 mL/min per 1.73 m^2 per year[5,18-21]. A community-based cohort study reported a decline in eGFR of 2.1 and 2.7 mL/min per 1.73 m^2 per year respectively for women and men with diabetes, whereas the rate of decline was 0.8 and 1.4 mL/min per 1.73 m^2 per year respectively for women and men without diabetes[18,22]. In subjects with CKD, a more rapid decline in renal function (ranging 1.03-4.3 mL/min per 1.73 m^2 per year) was noted[10,23-26] (Table 2). Some studies define rapid decline of eGFR in terms of absolute rate of loss, while others define it as percent change (Table 3)[3,27-30]. According to the Kidney Disease: Improving Global Outcomes (KDIGO) 2012 clinical practice guidelines for the evaluation and management of CKD, developed by the National Kidney Foundation, a rapid decline in renal function is defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year (as estimated using the 2009 CKD-EPI creatinine equation)[31]. It is generally believed that at present, there are a lack of well-controlled studies, which include frequent measurements and a long follow-up period, from which to establish an optimal definition of a rapid decline in renal function[18].

RISK FACTORS OF A RAPID DECLINE IN RENAL FUNCTION

An emerging challenge is the identification of potential factors associated with rapid renal function decline, which would form the basis for the development of strategies to prevent or retard disease progression, and reduce complications, thereby improving disease outcomes and quality of life in type 2 diabetics. Potential risk factors include ethnic/genetic and demographic factors, lifestyle and health behaviors, metabolic and biochemical abnormalities, cardiovascular functional factors, and some clinical symptoms of type 2 diabetes (Figure 1).

Table 1 Prevalence of albuminuria and impaired glomerular filtration rate in diabetic patients

Ref.	Population (Nationality)	Albuminuria prevalence	Impaired GFR prevalence
Parving *et al*[13]	International DEMAND study of 33 countries 2006 32208 type 2 diabetic patients	Microalbuminuria: 39% Macroalbuminuria: 10%	22%
Bos *et al*[108] data from: Herman *et al*[109]	Northern Africa Systematic review of PubMed 1990-2012	Egypt 1998: Albuminuria: 21%[109]	Egypt 1998-Outpatient clinics: 6.7%[109]
Hamed *et al*[111]	> 18 years old diabetic patients	Sudan 2008 (insulin treated diabetic patients): Albuminuria: 22%[110]	Egypt 1995-Hospital inpatients: 46.3%[111]
Icks A and Koch M Epidemiology of chronic kidney disease in diseases. In: Wolf G. Diabetes and Kidney Disease[11], data from: Chadban *et al*[112]	Australia AusDiab study: a national population-based cross-sectional survey > 25 years old diabetic patients	8.70% proteinuria-spot urine protein to creatinine ratio (abnormal: > 0.20 mg/mg)	27.60%
Unnikrishnan *et al*[113]	Southern India CURES 45 study 17, 16 type 2 diabetic patients	Microalbuminuria: 36.9% Macroalbuminuria: 2.2%	-
Icks A and Koch M Epidemiology of chronic kidney disease in diseases. In: Wolf G. Diabetes and Kidney Disease[11], data from: Lin *et al*[114]	Taiwan Community-based screening 1999–2001 > 30 years old type 2 diabetic patients	29.40% proteinuria-spot urine protein to creatinine ratio (abnormal: > 0.20 mg/mg)	15.10%
Yang *et al*[115]	China A nationally representative sample from 14 provinces and municipalities > 20 years old diabetic patients	17.30%	19.10%
Lou Arnal *et al*[116]	Spain A survey of 16 Health Centers of the Alcañiz Health Sector 2008 > 18 years old, 3466 type 2 diabetic patients	31.70%	25.20%
Detournay *et al*[117]	France ENTRED data 2007 A survey of the national public prescription claims database Type 2 diabetic patients	-	22%
Collins *et al*[14]	United Status NHANES study 2005-2010 Adult diabetic patients	29.90%	19.30%
Al-Rubeaan *et al*[54]	Saudi Arabia SNDR data > 25 yr, 54670 type 2 diabetic patients	Microalbuminuria: 1.2% Macroalbuminuria: 8.1%	GFR < 30 mL/min per 1.73 m^2: 1.50%

Albuminuria: Albumin-to-creatinine ratio (UACR) > 30 mg/g; Microalbuminuria: UACR 30-300 mg/g; Macroalbuminuria: UACR > 300 mg/g; Impaired glomerular filtration rate (GFR): Estimated GFR < 60 mL/min per 1.73 m^2; DEMAND: Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes study; AusDiab: The Australian Diabetes, Obesity and Lifestyle Study; CURES: Chennai Urban Rural Epidemiology Study; ENTRED: Échantillon national témoin représentatif des personnes diabétiques (National Representative Sample of Diabetic Patients); NHANES: National Health and Nutrition Examination Survey; SNDR: Saudi National Diabetes Registry.

Ethnic, genetic, and demographic factors

Ethnicity is a one of major factors affecting the progression of CKD in diabetic patients. In the United Kingdom, residents of South Asian origin had a higher prevalence of overt proteinuria and a lower prevalence of microalbuminuria compared to those with White European ethnicity[1,32]. In a 5-year retrospective, community-based cohort study of 135 general practices in East London, in which 3855 diabetic patients with an eGFR of < 60 mL/min per 1.73 m^2 were enrolled, renal function decline occurred at a significantly higher rate in South Asians as compared to other ethnicities[33]. According to the USRDS 2012 annual data report[34], ESRD caused by diabetes has increased in African-American, Native American, and Hispanic populations over the past decade[1,2,34]. USRDS 2013 also reported that the contribution of diabetes to ESRD was 59%-61% in Malaysia, Mexico, and Singapore in 2011, and above 40% in Israel, the Republic of Korea, Hong Kong, Taiwan, the Philippines, Japan, the United States, and New Zealand[14]. In summary, diabetic patients of Hispanic, black, Asian, and Maori ethnicity are at a higher risk of a rapid decline in renal function compared to white populations.

Ethnic differences in the presentation of diabetic kidney disease may reflect either genetic predisposition or differences in public health care policy[1], and thus, genetic studies need to exclude non-genetic confounders. Evidence of genes associated with diabetic nephropathy in type 2 diabetics comes mainly from family-based genome-wide linkage studies[35,36]. Findings from such studies include reports that 7p14.1 [engulfment and cell motility 1 (ELMO1)][37,38], 7q21.1/7q21.3[39] and 18q22.3

Table 2 Decline of estimated glomerular filtration rate in different populations

Population	eGFR decline (mL/min per 1.73 m^2 per year)	Ref.
Healthy		
PREVEND study 6894 subjects	0.55	Halbesma *et al*[5]
	Estimated using MDRD formula	
Annual health exam, Japan	0.36	Imai *et al*[19]
120727 subjects	Estimated using MDRD formula	
	modified by a Japanese coefficient	
ARIC study	0.47	Matsushita *et al*[20]
13029 subjects	Estimated using MDRD formula	
Tromso Study, Norway	1.21 (men)	Kronborg *et al*[21]
2249 men and 2192 women	1.19 (women)	
	Estimated using MDRD formula	
Aged without diabetes		
2475 men > 65 years old	1.4	Hemmelgarn *et al*[22]
3163 women > 65 years old	0.8	Hemmelgarn *et al*[22]
Aged with diabetes		
490 men > 65 years old	2.7	Hemmelgarn *et al*[22]
445 women > 65 years old	2.1	Hemmelgarn *et al*[22]
CKD		
MDRD study group	3.7	MDRD study group
eGFR 25-80 mL/min per 1.73 m^2, n = 28		Levey *et al*[23]
eGFR 7.5-24 mL/min per 1.73 m^2, n = 63	4.3	
African Americans with hypertension	2.21	Wright *et al*[24]
eGFR 20-65 mL/min per 1.73 m^2		
low mean arterial pressure, n = 380		
normal mean arterial pressure, n = 374	1.95	
Tromso Study, Norway	1.03	Eriksen *et al*[25]
eGFR 30-59 mL/min per 1.73 m^2		
3047 subjects		
eGFR < 60 mL/min per 1.73 m^2	2.65	Levin *et al*[26]
4231 subjects		

Data from Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group[18]; eGFR: Estimated glomerular filtration rate; CKD: Chronic kidney disease; PREVEND: Prevention of Renal and Vascular End-Stage Disease; ARIC: Atherosclerosis Risk in Communities; MDRD: Modification of Diet in Renal Disease Study.

[carnosine dipeptidase 1 (CNDP1)][40,41] are associated with the development of proteinuria and ESRD in African-Americans; 18q22.3 (CNDP1) is associated with proteinuria and ESRD in American-Indians[4]; and 17p14.1[37], 12q24.11 [acetyl-CoA carboxylase alpha (ACACB)][42], 13q34(rs1411766)[43], and 16q13 [solute-carrier group (SLC12A3)][44] may be associated with proteinuria and ESRD in Japanese[36]. Furthermore, haptoglobin (Hp) is a hemoglobin-binding protein that has a major role in protecting against heme-driven oxidative stress. Previous studies have shown the importance of the Hp genotype in the progression of diabetic nephropathy[45,46]. Moreover, diabetic patients with Hp 2-2 are more likely to develop nephropathy than those with Hp2-1 or Hp1-1[47,48].

Demographic factors may also influence the progression of diabetic kidney disease. Previous studies indicate that age is a significant predictor of progressive albuminuria and renal dysfunction in diabetics[49-54], and most studies reported that male sex is an important independent factor associated with renal function decline in type 2 diabetics[12,50,54,55]; however, some studies have shown an association with female sex[56].

Lifestyle and health behaviors

Smoking is an established factor for increased risk of development and rapid progression of diabetic kidney disease[12,54,57-59]. Also, some studies suggest an association between diet and renal function decline in diabetics, for example in those with high alcohol consumption[58] or a high-protein diet[59]. It has been demonstrated that a high dietary acid load (*e.g.*, in diets high in rice and meat) is associated with rapid progression of diabetic nephropathy to ESRD in Westernized South Asian people[60]. Lack of physical activity is also considered to be a risk factor in diabetic nephropathy[58], with a previous study reporting that high physical activity in women was associated with an improvement in eGFR[21].

Metabolic and biochemical factors

A number of metabolic conditions, such as hyperglycemia[61,62], dyslipidemia[63-65], or being overweight/obese[31,49,66], are widely recognized as being associated with the development of diabetic nephropathy, and are established factors in identifying subjects at a greater risk of disease progression[57]. Previous studies indicate that obesity, hyperglycemia, and dyslipidemia are significant predictors of progressive albuminuria[49-53,67,68]. A recent cross-sectional study reported UACR significantly correlated with metabolic syndrome and its components, including hyperglycemia, central obesity, and high triglyceride lev-

Table 3 Definitions of rapid renal function decline

	Population (Nationality)	Rapid renal function decline	Ref.
Study			
	United States 4380 patients from the community-based CHS ⩾ 65 years old Follow-up: 7 yr 14% with diabetes	> 3 mL/min per 1.73 m^2 per year	Reviewed by KDIGO CKD Work Group[18]: Shlipak *et al*[28] Rifkin *et al*[30]
	Taiwan 577 type 2 diabetes patients from an outpatient department in a hospital-based study 63 years old (mean age) Follow-up: 1 yr	> 3 mL/min per 1.73 m^2 per year	Sheen *et al*[72]
	472 CKD 4-5 patients from an outpatient department in a hospital-based study 65 years old (mean age) 35.4% with diabetes Follow-up: 1.5 yr (17.3 mo)		Tsai *et al*[118]
	Canada 4231 patients with eGFR < 30 mL/min per 1.73 m^2 from a cohort derived from all patients registered in a provincial database Follow-up: 2.5 yr (31 mo)	> 4 mL/min per 1.73 m^2 per year	Levin *et al*[26]
	Italy 1682 type 2 diabetes patients with eGFR ⩾ 60 mL/min per 1.73 m^2 from an outpatient department in a hospital based study Follow-up: 10 yr	> 4% per year	Zoppini *et al*[70]
	Canada 3154 patients with eGFR ⩾ 60 mL/min per 1.73 m^2, from the community based Walkerton Health Study (2002 to 2008) Follow-up: 7 yr	> 5% per year	Clark *et al*[74,119]
	Taiwan 7968 civil servants and teachers ⩾ 50 years old (mean age: 57 years old) Follow-up: 15 yr	> 20% per year	Reviewed by KDIGO CKD Work Group[18]: Cheng *et al*[29]
	Taiwan 167 patients in a hospital based study	> 25% per year	Chen *et al*[85]
Review	Chronic kidney disease Lancet	> 4 mL/min per 1.73 m^2 per year	Levey *et al*[3]
Guideline	KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease KDIGO CKD Work Group	> 5 mL/min per 1.73 m^2 per year	Inker *et al*[10] KDIGO CKD Work Group[18]

CHS: Cardiovascular Health Study; KDIGO: Kidney Disease: Improving Global Outcomes; eGFR: Estimated glomerular filtration rate; CKD: Chronic kidney disease.

els[65,69]. Factors associated with eGFR decline and progressive albuminuria might overlap. During a 10-year follow-up, an observational study of 1682 type 2 diabetics with baseline eGFR ⩾ 60 mL/min per 1.73 m^2 reported that obese patients had a significantly faster age-adjusted annual eGFR decline[70]. A positive association between glycated hemoglobin (HbA1c) and CKD has also been observed in type 2 diabetics, even in the absence of albuminuria and retinopathy[52]. An association between blood glucose, low-density lipoprotein abnormalities, and the progression of renal damage in diabetes has been reported[71]. HbA1c was found to be independently associated with rapid renal function decline in a group of type 2 diabetics without symptomatic cardiovascular disease[72].

Albuminuria and eGFR are not only biomarkers for the diagnosis and categorization of CKD[4], but are also well-known predictors of renal function decline, ESRD, and death in type 2 diabetics[8,73]. Proteinuria is associated with rapid decline in renal function[49-53], and a previous study suggests that dipstick proteinuria measurement could be used as a screening tool for rapid renal function decline[74].

Abnormalities in cardiovascular function

CKD shares many risk factors with cardiovascular disease[72,75], and dysfunction in one system can often lead to dysfunction in the other[49]. In patients with concomitant hypertension and type 2 diabetes, the risk of progression to ESRD is 7 fold that for age-matched control subjects[49,76]. Hypertension is a significant risk factor for insufficient renal function, cardiovascular events, and death in patients both with and without type 2 diabetes[49,61,77-79]. Previous studies show that systolic blood pressure (SBP) and pulse pressure are stronger predictors than diastolic blood pressure of renal outcomes, and are independent risk factors in the rapid decline of eGFR in type 2 diabet-

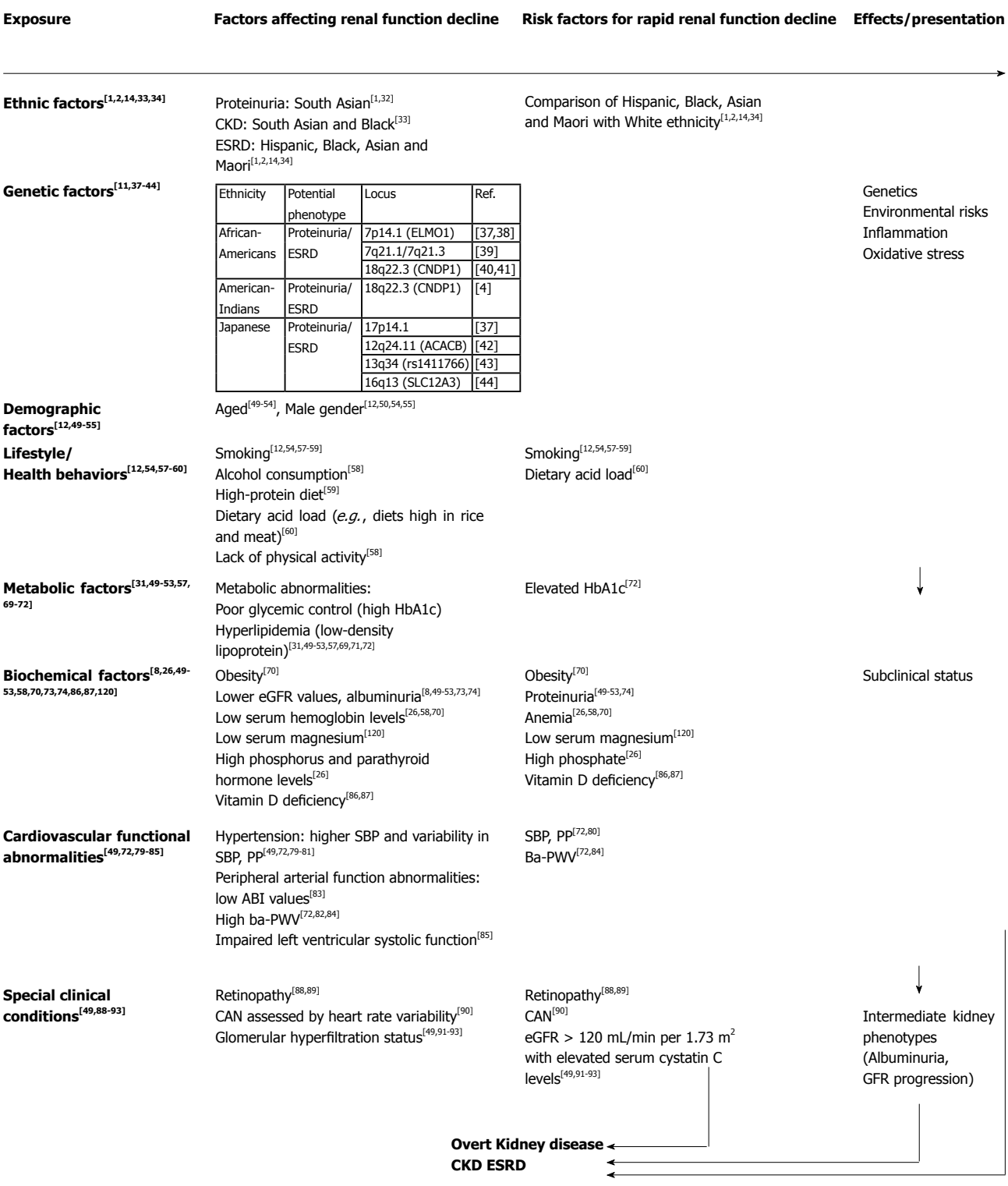

Figure 1 Conceptual model for diabetic kidney disease and potential risk factors of rapid renal function decline. CKD: Chronic kidney disease; HbA1c: Glycated hemoglobin; ESRD: End stage renal disease; eGFR: Estimated glomerular filtration rate; SBP: Systolic blood pressure; ba-PWV: Brachial-ankle pulse-wave velocity; PP: Pulse pressure; CAN: Cardiac autonomic neuropathy; ELMO1: Engulfment and cell motility 1; CNDP1: Carnosine dipeptidase 1; *ACACA*: *Acetyl-CoA carboxylase alpha*; *rs*: *RefSNP (Single Nucleotide Polymorphism) numbers*; SLC: Solute-carrier group.

ics[72,80], while another study suggests that both SBP and variability in SBP are risk factors in the development and progression of diabetic nephropathy[81].

In addition to blood pressure, peripheral arterial functional markers are also associated with renal function in type 2 diabetics[82]. A low ankle-brachial index was found to be significantly associated with a low eGFR[83]. Also, arterial stiffness is associated with incident albuminuria and decreased eGFR[72,84], and brachial-ankle pulse-wave velocity (ba-PWV) values are independently associated with rapid renal function decline in type 2 diabetics without symptomatic cardiovascular disease[72]. One study

reports that impaired left ventricular systolic function and increased ba-PWV are independently associated with a rapid decline in renal function[85].

Miscellaneous

Some other factors, such as low hemoglobin levels and electrolyte imbalance, may cause a rapid progression in diabetic kidney disease. Conditions including anemia, low serum magnesium levels, and high phosphorous and parathyroid hormone levels, are associated with rapid renal function decline in type 2 diabetics[26,58,70]. Furthermore, vitamin D deficiency associated with albuminuria was an independent risk factor in diabetic nephropathy after adjusting for demographic factors, hypertension, dyslipidemia, smoking status, and medication use[86,87].

Type 2 diabetic patients with additional microvascular complications, such as retinopathy or neuropathy, may also experience a rapid decline in renal function. Several studies have demonstrated that the rate of renal disease progression in type 2 diabetics with retinopathy is faster than that observed in those without retinopathy[88,89]; thus, screening for retinopathy may be helpful in identifying high-risk patients. Another study on cardiac autonomic neuropathy that assessed heart rate variability suggests that this is also an independent predictor of eGFR decline and could also be used as an identifying factor[90].

Special issues

Glomerular hyperfiltration and rapid renal function decline in type 2 diabetes: A longitudinal study of 600 type 2 diabetics with albuminuria < 200 μg/min, found that those with an eGFR > 120 mL/min per 1.73 m^2 had a higher risk of albuminuria progression (hazard ratio: 2.16) compared with those without baseline hyperfiltration; over a 4-year follow-up, renal function decline was relatively rapid, at an annual rate of up to 3.37 mL/min per 1.73 m^2[91]. Another study evaluated type 2 diabetic Pima Indians selected from participants in the Diabetic Renal Disease Study, with a baseline iothalamate clearance above the median for the entire study cohort (120 mL/min per 1.73 m^2) to give a study group with a normal or elevated GFR[92]. After a mean follow-up of 3.8 years, it was shown that directly measured GFR declined at 4.4% per year, and supposed that an increase in serum cystatin C provide means for detecting early renal function decline in diabetes[92]. Measurement of serum cystatin C may help to identify groups at high risk of renal function decline based on hyperfiltration status[49,93].

Non-albuminuric diabetic kidney disease: Renal insufficiency in the absence of albuminuria in patients with type 2 diabetes is another issue that should be noted. In a 1977 study of type 2 diabetic adults, 13% had an eGFR < 60 mL/min per 1.73 m^2, and 30% had neither albuminuria nor retinopathy[94]. Furthermore, data from UKPDS[12], DEMAND[13], and Atherosclerosis risk in Communities (ARIC)[52] studies suggests that the occurrence of renal impairment in type 2 diabetics without albuminuria is not unusual[49]. Microalbuminuria and reduced eGFR have been suggested as markers of different pathologic processes, with microalbuminuria associated with endothelial dysfunction and reduced eGFR being a renal manifestation of systemic atherosclerosis[49,95]. These patients are at higher risk of CKD progression, as the absence of proteinuria may lead to delays in the diagnosis and treatment of diabetic nephropathy[1,49].

POSSIBLE MANAGEMENT STRATEGIES

A number of therapeutic interventions for diabetic kidney disease have been developed over the past few decades[96]. Several studies have demonstrated increased activity in the renin-angiotensin-aldosterone system in diabetic patients with nephropathy[97,98]. Angiotensin-converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) treatment for diabetics with hypertension can reduce renal damage and may reduce cardiovascular complications[97-99]; thus, ACEI or ARB are recommended as a first-line treatment for diabetics with hypertension[2,10,98,100,101]. However, based on the ONTARGET trial, acute dialysis, hyperkalemia, and hypotension tended to be more frequent with the use of both ACEI and ARB; thus, dual inhibition of the renin-angiotensin system is not recommended[102]. Primary multifactorial interventions aimed at slowing progression of diabetic nephropathy include combination therapy targeting hyperglycemia, hypertension, microalbuminuria, and dyslipidemia[59]. The Steno-2 study, of 151 type 2 diabetics with baseline microalbuminuria who underwent multifactorial treatment, reported that at a 7.8-year follow-up 46 patients showed remission to normoalbuminuria, improved hypertensive and glycaemic control were independent predictors for remission, and that kidney function may have been preserved through a slower rate of eGFR decline[103]. Other studies provide evidence that intensive multifactorial management is more effective than conventional treatment[104-107]. In addition to blood pressure, glycemic and lipid control, lifestyle modifications such as cessation of smoking, protein restriction in diets, weight reduction[2,59], light to moderate exercise[4], and vitamin C[104,105] and vitamin D supplementation[26], may be helpful in preventing or slowing the progression of diabetic kidney disease[2,26,59].

CONCLUSION

The progression of diabetic kidney disease is highly variable. According to the KDIGO 2012 clinical practice guidelines for the evaluation and management of CKD, a rapid decline in renal function was defined as a sustained decline in eGFR of > 5 mL/min per 1.73 m^2 per year. Associated risk factors in patients with type 2 diabetes include ethnic/genetic and demographic factors, lifestyle and health behaviors, advanced albuminuria, poor glycemic control, dyslipidemia, and some biochemical abnormalities. Diabetic patients with retinopathy or cardiac

autonomic neuropathy are at increased risk of a rapid decline in eGFR. Furthermore, those with glomerular hyperfiltration and elevated serum cystatin C may also be at increased risk of a rapid decline in renal function. Early detection of high-risk groups with a more aggressive multifactorial approach to renal and cardiovascular protection is important.

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P- Reviewer: Nakhoul FM, Tamemoto H **S- Editor**: Song XX
L- Editor: A **E- Editor**: Lu YJ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Place of sodium-glucose co-transporter type 2 inhibitors for treatment of type 2 diabetes

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Received: July 2, 2014 Revised: July 19, 2014
Accepted: August 27, 2014
Published online: December 15, 2014

Abstract

Inhibitors of sodium-glucose co-transporter type 2 (SGLT2), such as canagliflozin and dapagliflozin, are recently approved for treatment of type 2 diabetes. These agents lower blood glucose mainly by increasing urinary glucose excretion. Compared with placebo, SGLT2 inhibitors reduce hemoglobin A1c (HbA1c) levels by an average of 0.5%-0.8% when used as monotherapy or add-on therapy. Advantages of this drug class include modest weight loss of approximately 2 kg, low risk of hypoglycemia, and decrease blood pressure of approximately 4 mmHg systolic and 2 mmHg diastolic. These characteristics make these agents potential add-on therapy in patients with HbA1c levels close to 7%-8.0%, particularly if these patients are obese, hypertensive, and/or prone for hypoglycemia. Meanwhile, these drugs are limited by high frequency of genital mycotic infections. Less common adverse effects include urinary tract infections, hypotension, dizziness, and worsening renal function. SGLT2 inhibitors should be used with caution in the elderly because of increased adverse effects, and should not be used in chronic kidney disease due to decreased or lack of efficacy and nephrotoxicity. Overall, SGLT2 inhibitors are useful addition for treatment of select groups of patients with type 2 diabetes, but their efficacy and safety need to be established in long-term clinical trials.

Key words: Type 2 diabetes; Canagliflozin dapagliflozin; Weight loss; Hypoglycemia; Chronic kidney disease; Genital infection

Core tip: Sodium-glucose co-transporter type 2 inhibitors are recently approved drugs for type 2 diabetes with unique mechanism of action. In this minireview, the author provides a practical approach on how to select the best candidates for these drugs.

Original sources: Mikhail N. Place of sodium-glucose co-transporter type 2 inhibitors for treatment of type 2 diabetes. *World J Diabetes* 2014; 5(6): 854-859 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/854.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.854

INTRODUCTION

In healthy individuals, almost all glucose filtered by the kidneys is reabsorbed into the circulation, and less than 0.5 g of glucose per day is lost in urine[1]. Ninety per cent of glucose reabsorption from glomerular filtrate is mediated by sodium-glucose co-transporter type 2 (SGLT2) located in early segments (called S1 and S2) of proximal renal tubules[2,5]. The remaining 10% of filtered glucose is reabsorbed by means of SGLT1 located in late segment (S3) of proximal tubule[3]. SGLT2 inhibitors decrease hyperglycemia independently of insulin by lowering the renal threshold for glucose and therefore increasing urinary excretion of glucose[2]. Canagliflozin (Invokana) is the first SGLT2 inhibitor approved in the United States in March 2013 for treatment of type 2 diabetes[4]. Dapagliflozin

Table 1 Differences between canagliflozin and dapagliflozin

	Canagliflozin (Invokana)[4]	Dapagliflozin (Forxiga)[5]
Approved doses	Starting dose 100 mg tablet qd, taken before breakfast. If tolerated, dose can be increased to 300 mg tablet *qd*	Starting dose 5 mg tablet qd taken in the morning with or without food. If tolerated, dose can be increased to 10 mg tablet *qd*
Use in CKD	Contraindicated with eGFR < 45 mL/min per 1.73 m^2. Dose limited to 100 mg/d with eGFR of 45-59 mL/min per 1.73 m^2	Not recommended with eGFR < 60 mL/min per 1.73 m^2. No dose adjustment is needed with milder CKD
Hepatic impairment (Child-Pugh classification: A: mild, B: moderate, C: severe)	No dosage adjustment is needed with mild or moderate hepatic impairment. Not recommended with severe hepatic impairment	No dosage adjustment is needed with mild or moderate hepatic impairment. Start with smaller dose (5 mg/d) in severe hepatic impairment then the high-dose 10 mg/d if tolerated
Drug interactions	Use higher dose (300 mg/d) with UGT enzyme inducers (*e.g.*, rifampin) ↑ C max of digoxin by 36%. Use low starting digoxin doses, and monitor serum digoxin levels closely	No dose adjustment is needed when used with UGT enzyme inducers No interaction with digoxin
Effect on LDL-C levels (mean percentage change *vs* placebo)	↑ 4.5%-8%	↑ 3.9%
Possible increase in cardiovascular events	A trend toward increase in non fatal stroke and cardiovascular events (see text)	Not observed
Possible increase in cancer	Not observed	Possible increase in bladder cancer (0.17% *vs* 0.03% with placebo)

eGFR: Estimated glomerular filtration rate; Cmax: Maximum plasma concentration; CKD: Chronic kidney disease.

(Forxiga) was approved by the European Medicines Agency in November 2012, and by the Federal Drug Administration (FDA) in the United States in January 2014[5]. While head to head trials are lacking, some important differences exist between canagliflozin and dapagliflozin (Table 1). Many SGLT2 inhibitors such as empagliflozin, ipragliflozin, luseogliflozin are pending approval or still under development[2,6]. The main purpose of this review is to identify the optimum place of SGLT2 inhibitors in management of patients with type 2 diabetes based on both patients' characteristics and drug profile of SGLT2 inhibitors. More emphasis will be placed on the 2 approved SGLT2 inhibitors: canagliflozin and dapagliflozin.

SEARCH METHODOLOGY

PubMed search was conducted until July 2014 to identify all humans studies related to efficacy and safety of all SGLT2 inhibitors published in the English, Spanish and French literature. The search included all clinical trials of various SGLT2 inhibitors, pertinent guidelines of experts, review articles, prescribing information of canagliflozin and dapagliflozin are also reviewed. Search terms included "sodium glucose co-transporters", "diabetes mellitus", "canagliflozin", "dapagliflozin", "empagliflozin", "efficacy", "safety", "adverse effects", "cardiovascular effects", "mortality", "glycosuria".

Potential candidates for SGLT2 inhibitors

As add-on to other oral agents in patients with hemoglobin A1c levels of 7%-8.0%: In general, the efficacy of SGLT2 inhibitors is similar to metformin, sulfonylurea, pioglitazone, but canagliflozin may be slightly superior to sitagliptin [difference in hemoglobin A1c (HbA1c) 0.37%][7,8]. As result of their unique mechanism of action, SGLT2 inhibitors can be virtually combined with any other anti-diabetic therapy. A recent meta-analysis of 58 studies that included 8 different SGLT2 inhibitors showed that these agents reduced mean HbA1c levels by 0.79% when used as monotherapy and 0.61% when used as add-on treatment compared with placebo[7]. Because of universal agreement that metformin is the initial drug of choice for treatment of type 2 diabetes, the use of SGLT2 inhibitors as monotherapy is not justified except in selected patients who cannot tolerate metformin[9]. The place of SGLT2 inhibitors therefore is more appropriate as add-on therapy. For instance, after the addition of canagliflozin, dapagliflozin, and empagliflozin to patients with mean baseline HbA1c of approximately 8.0%, proportions of subjects who achieved HbA1c concentrations less than 7% were: 64% (*vs* 32% with placebo), 41% (*vs* 26% with placebo), and 32% (*vs* 9% with placebo), respectively[6,10,11]. In the previous 3 trials, background diabetes treatment consisted of metformin + pioglitazone, metformin alone, and metformin + sulfonylurea, respectively[6,10,11]. Clearly, in these studies, not all subjects achieved the HbA1c target of less than 7%. Hence, as baseline HbA1c levels become higher than 8.0% (*e.g.*, 8.5%-9%), the addition of a SGLT2 inhibitor may only improve, but unlikely optimize, glycemic control. In the latter setting, initiation of insulin is the most appropriate step.

Obese patients or patients concerned about weight gain: The use of SGLT2 inhibitors is consistently associated with mild weight loss of approximately 2 kg compared with placebo irrespective of presence or type of concomitant anti-diabetes therapy[7]. Weight loss becomes evident after 6 wk then usually reaches a plateau or slight-

ly rebounds after 26-32 wk until the end of follow-up at 104 wk[12]. The main cause of weight loss is increased urinary glucose loss, estimated to be approximately 100 g of glucose per 24 h[13]. Since each gram of glucose excreted in urine translates into a loss of 4 kcal, a loss of approximately 400 kcal/d is expected with SGLT 2 inhibitors[14]. Two studies using dual-energy X-ray absorptiometry show that approximately two-thirds of the reduction in body weight associated with administration of dapagliflozin and canagliflozin originates from fat mass, whereas the remaining one third is derived from lean body mass[15,16]. Another contributing factor to weight reduction may be fluid loss as result of the diuretic action of SGLT2 inhibitors, particularly during the initial rapid decline in body weight[15]. Since weight gain is a major unwanted effect of insulin therapy, addition of a SGLT2 inhibitor was evaluated in obese patients receiving high insulin doses (77 units/d)[12]. Thus, patients randomized to dapagliflozin lost an average weight of 1.4 kg without changing insulin requirements. Conversely, subjects randomized to placebo gained 1.8 kg, and their insulin requirements increased by 18 units/d[12]. Moreover, the HbA1c levels were 0.4% lower among dapagliflozin-treated group *vs* the placebo group[12]. Therefore, in insulin-treated patients concerned about weight gain, addition of a SGLT2 inhibitor may be a viable option.

Patients prone for hypoglycemia: The use of SGLT2 inhibitors is associated with low risk for hypoglycemia that is generally similar or slightly greater than placebo[11], similar to metformin[17], but 7-11 times less common than sulfonylurea (SU)[16,18]. Thus, in one trial, hypoglycemia occurred in 5% of patients randomized to canagliflozin 300 mg/d *vs* 34% of patients randomized to glimepiride (mean maximum dose 5.6 mg/d)[16]. SGLT2 inhibitors can be therefore a reasonable alternative to SU in patients with frequent hypoglycemia. The low hypoglycemic risk of SGLT2 inhibitors is attributed to the fact that these agents reduce renal glucose threshold to a range close to 76-90 mg/dL, *i.e.*, level that is above the plasma glucose concentration at which hypoglycemic symptoms occur[13,14]. Meanwhile, the incidence of hypoglycemia associated with SGLT2 inhibitors may increase in 3 conditions namely concomitant therapy with insulin and/or SU, in chronic kidney disease (CKD), and in the elderly. Thus, when dapagliflozin 10 mg/d was added to a background of insulin therapy, frequency of hypoglycemia was numerically greater among patients randomized to dapagliflozin than placebo, 57% and 52%, respectively[19]. With respect to CKD, in one study of patients with estimated glomerular filtration rate (eGFR) between 30 and 49 mL/min per 1.73 m^2, the proportions of subjects with documented hypoglycemia were higher with both doses of canagliflozin being 52% *vs* 36% with placebo[20]. Of note, the vast majority (96%) of the previous study population was also taking insulin or SU[20]. Finally, regarding advanced age, in a study of older patients (mean age 64 years), the incidence of hypoglycemia was 36% and 28% with canagliflozin 300 mg/d, and placebo, respectively[21].

Patients with uncontrolled hypertension: In one meta-analysis of 27 randomized trials, the use of various SGLT2 inhibitors was associated with mean reduction of systolic and diastolic blood pressure of 4.0 mmHg and 1.6 mmHg, respectively compared with baseline[22]. Only canagliflozin showed dose-response relationship with systolic blood pressure[22]. The decrease in blood pressure is most likely due to osmotic diuresis, but mild weight loss may be another contributing factor[13]. It is reassuring that the decrease in blood pressure was not associated by an increase in heart rate[8,23].

Patients in whom SGLT2 inhibitors may be used with caution

Women with history of mycotic genital infections and uncircumcised men: Increased vaginal fungal infection is the most common adverse effect of SGLT2 inhibitors reported by 11%-14% of patients who received canagliflozin or dapagliflozin compared with 2%-4% in subjects randomized to placebo or a comparator agent such as glimepiride or sitagliptin[8,16]. The increased genetic mycotic infection is most likely related to the increase in urinary glucose excretion induced by SGLT2 inhibitors. The median time of diagnosis was 19 d after the initiation of canagliflozin, and the most frequently isolated Candida species were Candida albicans (51%) and Candida glabrata (37%)[24]. Infection is frequently recurrent, and patients with previous history of genital mycotic infections are more prone to develop this type of infection[4,19,25].

Increased frequency of genetic mycotic infections also occurs in men exposed to SGLT2 inhibitors, albeit to a lesser extent than in women[25]. These include balanitis or balanoposthitis. In the trial of Cefalu *et al*[16], frequency of genetic mycotic infections in men exposed to canagliflozin 100 mg/d, canagliflozin 300 mg/d, and glimepiride was 7%, 8%, and 1%, respectively. Rates of infection are relatively higher in uncircumcised men and those with history of balanitis[4,25]. In general, genital mycotic events in both genders were considered mild to moderate in severity, were treated with topical or oral anti-fungal agents without interruption of the drug, and uncommonly led to withdrawals[8]. The frequency of UTI is also increased with the use of SGLT2 inhibitors, being 7.2%, 5.1%, and 4.2% among patients randomized to canagliflozin 100 mg/d, 300 mg/d, and placebo, respectively[23]. Candida spp. was cultured from the urine specimens of 4.4% of canagliflozin-treated patients compared with 1.1% of control subjects[26]. This increased frequency of candiduria may reflect contamination from vaginal colonization[26].

Elderly patients: Two main reasons make the use of SGLT2 inhibitors in the elderly not an attractive option: diminished efficacy and increased frequency of some adverse effects. Thus, mean reduction of HbA1c with the highest dose of canagliflozin (300 mg/d) *vs* placebo was

0.8% and 0.5% after 26 wk among patients younger than 65 years and those who were older than 65 years, respectively[21]. This decreased efficacy was also demonstrated in a pooled analysis of 4 other canagliflozin studies[27]. Likewise, in one trial of dapagliflozin, reduction in HbA1c levels in patients younger than 65 (mean age 58 years) and older than 65 (mean age 70 years) was 0.4% and 0.3%, respectively after 24 wk compared with baseline[28]. Since the anti-hyperglycemic action of SGLT2 inhibitors rely on enhancing urinary glucose excretion, the decreased efficacy of the agents with old age is in large part attributed to the reduction in eGFR that normally occurs with aging[21]. Besides decreased efficacy, available data suggest that several adverse effects of SGLT2 inhibitors may increase with advanced age. First, elderly patients exposed to SGLT-2 inhibitors are more prone for worsening renal function than younger patients. Thus, in patients aged 65 and older, renal impairment and renal failure occurred among 14.8% of patients randomized to dapagliflozin *vs* 8.0% with placebo, whereas corresponding proportions in patients younger than 65 were 4.7% and 0.4%[28]. Second, elderly patients receiving canagliflozin and dapagliflozin may be more prone for volume-depletion adverse effects such as hypotension, dizziness, and syncopy[4,5,27]. Third, as mentioned earlier, elderly patients may be more susceptible to hypoglycemia associated with SGLT2 inhibitors[21].

Patients with significant history of vascular disease: The Canagliflozin Cardiovascular Assessment Study (CANVAS) is an ongoing large randomized trial that primarily examines the effects of canagliflozin on cardiovascular events and mortality in patients with long-standing type 2 diabetes and elevated cardiovascular risk[29]. An imbalance in the incidence of cardiovascular events was recorded during the first 30 d of CANVAS. Thus, 13 of 2889 patients had an event in the canagliflozin group compared with 1 of 1441 patients in the placebo group yielding a hazard ratio of 6.5 (95%CI: 0.85-49.6). This imbalance was not evident after 30 d[7]. In addition, the FDA reported a trend toward an increase in nonfatal stroke in patients who received canagliflozin [HR = 1.46 (95%CI: 0.83-2.58)][7]. Regarding dapagliflozin, the limited available data is somewhat reassuring. Thus, one trial of older patients (mean age 64 years) with advanced type 2 diabetes and history of cardiovascular disease did not show difference in cardiovascular events or mortality between patients randomized to dapagliflozin compared to placebo after 52 wk of intervention[28].

Patients with osteoporosis: Incidence rate of bone fractures derived from pooled data of 8 trials were 18.7, 17.6, and 14.2 per 1000 patient years of exposure to canagliflozin 100 mg/d, 300 mg/d, and comparator, respectively[4]. In one study of patients with moderate renal impairment (mean age 67 years), 13 of 85 (7.7%) patients randomized to dapagliflozin experienced fracture compared to none of the 84 subjects randomized to placebo[30]. The reasons of excess fractures in patients exposed to canagliflozin and dapagliflozin are unclear. No notable changes in serum or urine calcium, 1.25 dihydroxy vitamin D, or parathyroid hormone were reported[19]. However, in 2 canagliflozin studies, there was a modest increase in one marker of bone resorption, serum collagen type 1 β-carboxy-terminal telopeptide[14,21]. Nevertheless, until further data become available, SGLT2 inhibitors should be used with caution in patients having history of osteoporosis or fractures.

Patients in whom SGLT2 inhibitors should be avoided

Patients with chronic kidney disease: As mentioned earlier, the risk of hypoglycemia associated with the use of SGLT2 inhibitors in patients with CKD is increased[20]. Other reasons to avoid the use of these drugs in CKD are decreased or lack of efficacy and worsening renal function. Thus, in patients with stage 3 CKD, defined as eGFR between 30 and 49 mL/min per 1.73 m^2, the efficacy of canagliflozin was only modest with mean HbA1c reduction of 0.4% as compared with placebo[20]. Furthermore, in another trial of patients with eGFR of 30 to 59 mL/min per 1.73 m^2, dapagliflozin did not have any significant effect on HbA1c levels compared with placebo[30]. This decreased or absent efficacy of SGLT2 inhibitors in CKD is most likely the result of reduction of renal glucose clearance as eGFR declines[21,31]. Patients with CKD are particularly susceptible to the nephrotoxic effects of SGLT2 inhibitors. Indeed, increase in serum creatinine, and decrease in eGFR were demonstrated after 1-3 wk of exposure to dapagliflozin and canagliflozin, respectively[20,30]. Therefore, the use of dapagliflozin and canagliflozin is contraindicated in patients with eGFR < 60 mL/min per 1.73 m^2, and 45 mL/min per 1.73 m^2, respectively[4,5].

Patients with high low density lipoprotein-cholesterol (LDL-C) concentrations: For unclear reason, canagliflozin was found to increase plasma levels of LDL-C in a dose-related fashion. In pooled data from 4 placebo-controlled trials, mean percentage increases over baseline values were 4.5% and 8% with 100 mg/d and 300 mg/d, respectively relative to placebo[4]. In one study of 26 wk-duration, slight increases in plasma levels of apolipoprotein B of 1.2% and 3.5% were reported among patients randomized to canagliflozin 100 mg/d, and 300 mg/d, respectively compared with 0.9% increase with placebo[23]. Canagliflozin also increased levels of high density lipoprotein-cholesterol, with mean percentage increase of 6.1%-6.8% relative to placebo[23], and 8%-9% relative to glimepiride[16]. Clearly, the increase in plasma levels of LDL-C and apolipoprotein B is concerning, and its impact on cardiovascular events needs to be carefully examined. The effect of dapagliflozin on LDL-C levels is inconsistent. In pooled data from 13 placebo-controlled trials, mean percentage increase in LDL-C levels was 2.9% in dapagliflozin groups *vs* -1% in placebo groups after 24 wk[5]. Yet, in one trial lasting 2 years, no change in LDL-C

levels was recorded in dapagliflozin-treated subjects[12].

Patients with history of bladder cancer: Possible increased risk of bladder cancer was observed in dapagliflozin trials[5]. Accordingly, dapagliflozin should not be used in patients with history of bladder cancer until further data become available[5].

OTHER LIMITATIONS OF SGLT2 INHIBITORS

Although almost all clinical trials of SGLT2 inhibitors are randomized and double-blind, they are sponsored by corresponding manufacturers, and therefore open to various bias, *e.g.*, using comparator drug in submaximal doses, or not mentioning its actual doses[16,18]. Moreover, the meta-analysis of Vasilakou *et al*[7] revealed that reduction in HbA1c levels by these agents may be overstated because of high discontinuation rates and handling missing data by the use of "last observation carried forward". Indeed, the latter method is considered inappropriate and can potentially inflate drug efficacy[32]. The high cost, and absence of long-term data (*e.g.*, 5 years or more) are further limitations of this new class of drugs.

CONCLUSION

Owing to their unique mechanism of action and acceptable efficacy, SGLT2 inhibitors represent a useful add-on therapy in patients with uncontrolled type 2 diabetes. Patient subgroups that would potentially benefit the most from this class are those with HbA1c levels in the range of 7%-8%, subjects concerned about weight gain, patients prone for hypoglycemia, or those with uncontrolled hypertension. On the other hand, these agents are not recommended in CKD, and should be used with caution in the elderly. It may be wise not to use canagliflozin in patients with established cardiovascular disease and high LDL-C levels until further data become available. The results of the ongoing large randomized trials should clarify the long-term safety of different members of SGLT2 inhibitors with respect to cardiovascular morbidity and mortality, incidence of cancer and fractures[29,33].

ACKNOWLEDGMENTS

The author thanks the librarian Irene Lovas, MLS, for her expert help with the literature review.

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P- Reviewer: Kawalec P, Ozdemir S **S- Editor**: Song XX
L- Editor: A **E- Editor**: Lu YJ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Type 2 diabetes mellitus and Alzheimer's disease

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Received: August 12, 2014 Revised: October 17, 2014
Accepted: October 31, 2014
Published online: December 15, 2014

Abstract

Epidemiological and biological evidences support a link between type 2 diabetes mellitus (DM2) and Alzheimer's disease (AD). Persons with diabetes have a higher incidence of cognitive decline and an increased risk of developing all types of dementia. Cognitive deficits in persons with diabetes mainly affect the areas of psychomotor efficiency, attention, learning and memory, mental flexibility and speed, and executive function. The strong epidemiological association has suggested the existence of a physiopathological link. The determinants of the accelerated cognitive decline in DM2, however, are less clear. Increased cortical and subcortical atrophy have been evidenced after controlling for diabetic vascular disease and inadequate cerebral circulation. Most recent studies have focused on the role of insulin and insulin resistance as possible links between diabetes and AD. Disturbances in brain insulin signaling mechanisms may contribute to the molecular, biochemical, and histopathological lesions in AD. Hyperglycemia itself is a risk factor for cognitive dysfunction and dementia. Hypoglycemia may also have deleterious effects on cognitive function. Recurrent symptomatic and asymptomatic hypoglycemic episodes have been suggested to cause sub-clinical brain damage, and permanent cognitive impairment. Future trials are required to clarify the mechanistic link, to address the question whether cognitive decline may be prevented by an adequate metabolic control, and to elucidate the role of drugs that may cause hypoglycemic episodes.

Key words: Dementia; Alzheimer; Type 2 diabetes; Aging; Cognitive decline; Mild cognitive impairment; Insulin; Hypoglycemia; Hyperglycemia

Core tip: Epidemiological and biological evidences support a link between type 2 diabetes (DM2) and Alzheimer's disease (AD). Persons with diabetes have increased incidence of cognitive decline and AD. Increased cortical and subcortical atrophy is present after controlling for vascular disease and inadequate cerebral circulation. Recent studies confirmed the role of insulin as possible link between DM2 and AD. Altered insulin signaling may contribute to AD biochemical and histopathological lesions. Hyperglycemia and hypoglycemia also have deleterious effects on cognitive function. Future trials would clarify the mechanistic link, and if cognitive decline may be prevented by an adequate metabolic control, and avoiding hypoglycemia.

Original sources: Barbagallo M, Dominguez LJ. Type 2 diabetes mellitus and Alzheimer's disease. *World J Diabetes* 2014; 5(6): 889-893 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/889.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.889

INTRODUCTION

Type 2 diabetes mellitus (DM2) and Alzheimer's disease (AD) are age-related conditions, both characterized by increased incidence and prevalence with aging[1,2].

DM2 is one of the fastest growing epidemics at present, which is frequently associated with aging. Characteristic features of DM2 include impairments in insulin actions

and signaling. Insulin resistance in peripheral tissues results in hyperglycemia and hyperinsulinemia. AD is the most common neurodegenerative disorder, and its incidence increases with age[3]. AD is characterized by the presence of several pathological hallmarks including neuronal loss, formation of senile plaques composed by extracellular deposits of amyloid beta, intracellular neurofibrillary tangles composed of aggregated hyperphosphorylated tau proteins in brain, proliferation of astrocytes, and activation of microglia. These features are accompanied by mitochondrial dysfunction and alterations in neuronal synapses[3]. The molecular and pathophysiological mechanisms that underlie AD still have many dark sides. Although etiology and the exact mechanism that trigger the pathological alterations of AD are still not clear, most studies have suggested that the deposit of the toxic amyloid-beta peptide caused by an abnormal processing of amyloid-beta precursor protein (amyloid cascade hypothesis), may initiate and/or contribute to the pathogenesis of AD.

EPIDEMIOLOGICAL EVIDENCES

Mounting epidemiological and biological evidences support a link between these two aging related diseases. First and foremost, diabetes mellitus is associated with changes in cognition, and cognitive dysfunction.

Persons with diabetes have been reported to hold a higher incidence of cognitive decline and AD; DM2 has been strongly associated with an increased risk of developing all types of dementia, including AD[2,4-6]. A systematic review including fourteen eligible longitudinal population-based studies of variable methodological quality found that in most studies the incidence of "any dementia" was higher in persons with diabetes than in those without diabetes[7]. Although, in some studies there are methodological limitations, the association remains strong. Some studies have relied on self-reported diagnosis of diabetes, and in the elderly population many patients with diabetes may remain undiagnosed. For the same reason, the duration of diabetes is also difficult to ascertain in older adults[8].

In a longitudinal cohort study, lasting up to 9 years, the risk of developing Alzheimer's disease was 65% higher in persons with diabetes than in non-diabetic controls[9]. In a community-based controlled study (Mayo Clinic Alzheimer Disease Patient Registry) the prevalence of diabetes and glucose intolerance was examined in patients with AD *vs* control participants without AD. The study suggested that frank diabetes (35%) or glucose intolerance (46%) might be present in up to 80% of patients with AD[10].

Even with the limitations discussed above, several studies have suggested that longer diabetes duration is generally associated with a higher risk for developing dementia[6,11,12]. In random effects models, DM2 was associated with lower levels of global cognition, episodic, semantic and working memory, and visuospatial ability at baseline[9]. Cognitive deficits in DM2 mainly affected the areas of psychomotor efficiency, attention, learning and memory, mental flexibility, and speed and executive function[13,14].

Recent studies have also shown a positive association between DM2 and mild cognitive impairment (MCI), and an accelerated progression from MCI to dementia in DM2[15]. A retrospective case-notes review of people with known diabetes who were resident in nursing homes in England showed very significant levels of disability and comorbidity, and in this setting, dementia was the most common comorbidity[16].

PHYSIOPATHOLOGICAL LINK

The strong epidemiological association has suggested the existence of a physiopathological link. However, the determinants of the accelerated cognitive decline in DM2 are less clear. The most studied hypothesis proposes that the primary cause of the association may be linked to the diabetic vascular disease and inadequate cerebral circulation, with subsequent silent ischemic damage induced by diabetes. However, even after controlling for cardiovascular risk factors, several studies on the cerebral structure of patients with diabetes have evidenced increased cortical and subcortical atrophy, besides increased leukoaraiosis, which were associated with impaired cognitive performance[17,18].

Most recent studies have focused on the possible role of insulin, and insulin action. Insulin resistance has been strongly implicated as a possible link between DM2 and AD. A condition of hyperinsulinemia, regardless of the presence of DM2, appears to be associated with a worse cognitive performance. There is a rapid growth in the literature pointing toward insulin deficiency and insulin resistance as mediators of AD-type neurodegeneration. De la Monte has even suggested that AD may be termed as "type 3 diabetes", indicating that AD may represent a form of diabetes that selectively involves the brain with molecular and biochemical features that overlap with diabetes mellitus[19].

The importance of the role of insulin in brain aging has long been known. Insulin has significant neurothrophic properties in the brain. The hormone is rapidly transported to the level of the central nervous system through the blood-brain barrier by a transport mechanism mediated by insulin receptors. It is interesting to note that these receptors are mainly localized at the level of the hippocampus, entorhinal cortex and frontal areas known to be involved in functions such as memory and learning. Insulin is also involved in the production of important neurotransmitters such as acetylcholine and norepinephrine. It is known that an acute increase in circulating levels of insulin, as it occurs in the post-prandial period, determines a physiological parallel increase of the concentrations of the hormone in the brain. A state of chronic hyperinsulinemia, as it occurs in insulin-resistance conditions and in DM2 may

determine a down-regulation of the insulin receptors at the blood-brain barrier, thus reducing the transport of insulin in the brain. Evidence is growing to link an alteration of metabolism and the deposition of precursors of amyloid in the brain that may occur in persons with diabetes, which is suggested as the pathogenesis of AD in DM2. The amyloid precursor protein is a transmembrane protein consisting of 770 amino acids; it is known to be the precursor of the amyloid beta involved in the etiopathogenesis of AD. Although the role of amyloid beta and its isoforms has yet to be elucidated, it seems to take part in numerous physiological processes. How can clinical hyperinsulinemia be a risk factor for AD even if insulin is an important neurothrophic factor? These two apparent paradoxal findings may be reconciled by the notion of insulin resistance. Whereas insulin is a neurothrophic factor at moderate concentrations, hyperinsulinemia with elevated concentrations of insulin in the brain may be associated with reduced amyloid-beta clearance due to competition for their common and main degrading mechanism-the "Insulin-Degrading Enzyme" (IDE). Insulin modulates metabolism of amyloid precursor protein decreasing intracellular accumulation. Insulin is degraded by the IDE, which is also involved in the metabolism and degradation of amyloid beta. This multifunctional enzyme degrades insulin and amylin, peptides related to the pathology of DM2, together with amyloid-beta peptide in the AD brain. Hyperinsulinemia may elevate amyloid beta through insulin's competition with amyloid beta for IDE[20]. Therefore, it has been suggested that the link between hyperinsulinemia and AD may be the IDE. Since IDE is much more selective for insulin than for amyloid beta, brain hyperinsulinemia may deprive amyloid beta of its main clearance mechanism, favoring its accumulation in the brain, and its consequent neurotoxic effects[21].

Disturbances in brain insulin signaling mechanisms represent early and progressive abnormalities and could account for the majority of molecular, biochemical, and histopathological lesions in AD. Increasing insulin resistance and hyperinsulinemia were associated with more hippocampal and amygdalar atrophy on magnetic resonance imaging (MRI) in persons with DM2 when compared to matched non-diabetic controls, regardless of vascular pathology[13,17]. Given these links, it has been suggested that may be a common underlying mechanism predisposes to amyloid deposition in the brain and in the pancreatic islet[10].

Glucose levels itself are a risk factor for cognitive dysfunction and dementia. In a prospective, community-based cohort study, higher plasma glucose concentrations were associated with an increased risk of dementia in populations with and without diabetes, suggesting that higher levels of glucose may have deleterious effects on the aging brain[22].

Although there is still limited knowledge concerning the association between impaired fasting glucose and/or impaired glucose tolerance and cognitive impairment, there is increasing evidence that these prediabetic conditions may increase the risk of AD in elderly patients. The risk of incident dementia increased in diabetic and in non-diabetic persons according to the average glucose concentrations during the preceding 5 years[22]. Hyperglycemia and hyperinsulinemia may accelerate brain aging also by inducing *tau* hyperphosphorylation and amyloid oligomerization, as well as by leading to widespread brain microangiopathy. Persons with diabetes are more prone to develop accelerated leukoaraiosis (white matter high-intensity lesions)[23].

GLYCEMIC CONTROL AND THE ROLE OF HYPOGLYCEMIA

The effect of diabetes treatment and glycemic control on dementia risk are less clear. It has been suggested that glycemic control may have a role in preserving cognitive performance among patients with DM2. Using baseline cognitive measures collected in the Memory in Diabetes, sub-study of the Action to Control Cardiovascular Risk in Diabetes trial, the authors found that a 1% higher glycated hemoglobin A (HbA1c) value was associated with a significant lower test performance and memory score in patients with diabetes[24].

HbA1c was also identified as an additional risk factor for a greater rate of brain atrophy. Enzinger *et al*[25], measuring the annual brain volume changes over 6 years with MRI in 201 participants in the Austrian Stroke Prevention Study, found significant differences in brain atrophy rates by quartiles of HbA1c levels[25]. Clustering of factors associated with the so-called metabolic syndrome in persons with high HbA1c suggests a link between this syndrome, which is associated with insulin resistance and hyperinsulinemia, with late-life brain tissue loss[25]. In diabetic patients, an inverse relationship was found between serum HbA1c and working memory, executive functioning, learning, and complex psychomotor performance, supporting the hypothesis that an inadequate glucose control may be associated with worsening cognitive function[26,27].

However, an excessively tight glycemic control in older persons with DM2, and its related increased risk of hypoglycemia, may also have deleterious effects on cognitive function[28]. In the presence of hypoglycemia, several responses occur within the brain, including activation of the central sympathetic nervous system; hypoglycemic symptoms include alterations of cognitive function, such as difficulty in concentrating and drowsiness, among others. Recurrent symptomatic and asymptomatic hypoglycemic episodes have been suggested to cause sub-clinical brain damage, and permanent cognitive impairment[29]. In addition, hypoglycemic states may increase the action of the receptors through an arteriolar vasodilatation. Since chronic hyperglycemia in DM2 is associated with endothelial alterations[30], this may cause in case of hypoglycemia a reduced vasodilating effect at the level of the blood-brain barrier, with a possible amplification of the brain

damage due to hypoglycemia itself. Among older patients with type 2 diabetes, a history of severe hypoglycemic episodes collected and reviewed using hospital discharge and emergency department diagnoses from 1980-2002 was associated with a greater risk of dementia[31]. More recently, a 12 years prospective population-based study of 783 older adults who were participating in the Health, Aging, and Body Composition Study, found a bidirectional association between hypoglycemia and dementia[32]. During the 12-year follow-up period, the participants who experienced at least one hypoglycemic event had a 2-fold increased risk for developing dementia, while older adults with DM2 who developed dementia had a greater risk for having a subsequent hypoglycemic event compared with participants who did not develop dementia[32].

Therefore, it has been suggested that drugs that cause lower postprandial glucose excursions and minor risk of hypoglycemia may prevent cognitive decline in older diabetic persons[33]. This data needs to be confirmed by future trials.

RESEARCH AND CLINICAL IMPLICATIONS

Cognitive function has not been included as an outcome in large scale randomized controlled trials of type 2 diabetes, and screening for dementia and cognitive impairment is still not included in routine diabetic patient care. There are sufficient epidemiological and clinical data to include an evaluation of cognitive complications in the clinical practice of persons with diabetes, in particular in those older than 70-75 years, and those with a long lasting history of diabetes.

There are some barriers in implementing a screening and diagnostic program for dementia in patients with diabetes. Neurocognitive testing in which an expert examiner administers a battery of tests to assess different aspects of cerebral function is still the gold standard for the diagnosis of dementia[14], and a computed tomography (CT) scan or an MRI may be required. This evaluation requires substantial financial and human resources. Screening cognitive tests are time consuming and CT scans are expensive[34]. However, diagnosis is even more important in older populations, because many older persons with diabetes nowadays live alone and self-manage their drugs. A mistake due to cognitive impairment may be extremely dangerous in particular in patients who need insulin, and self-practice insulin injections. Many hypoglycemic episodes may be due to errors in self-administration in undiagnosed subclinical demented patients.

CONCLUSION

There is convincing epidemiological evidence showing an increased risk of dementia in people with diabetes, but there are few mechanistic studies that provide a clear pathophysiological link, although the cause may be multifactorial. Cerebrovascular alterations, insulin action, insulin resistance, altered amyloid metabolism, chronic hyperglycemia, and recurrent hypoglycemic episodes seem to play a major role. Future trials are required to clarify the mechanistic link and to address the question whether cognitive decline may be prevented by an adequate metabolic control, and to better define the role of drugs that may cause hypoglycemic episodes. Clinicians treating older persons with diabetes should start to routinely search for cognitive impairment as well as they search for cardiovascular, renal, or other common complications of diabetic disease. There is sufficient evidence to support the view that time is probably arrived to incorporate cognitive evaluation in future national and international diabetic guidelines.

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P- Reviewer: Padwal RS **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Effect of periodontal treatment on adipokines in type 2 diabetes

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Received: August 26, 2014 Revised: September 29, 2014
Accepted: November 7, 2014
Published online: December 15, 2014

Abstract

The association between adipokines and inflammatory periodontal diseases has been studied over the last two decades. This review was intended to explore the observation that periodontal therapy may lead to an improvement of adipokines in diabetic patients. In summary, substantial evidence suggests that diabetes is associated with increased prevalence, extent and severity of periodontitis. Numerous mechanisms have been elucidated to explain the impact of diabetes on the periodontium. However, current knowledge concerning the role of major adipokines indicates only some of their associations with the pathogenesis of periodontitis in type 2 diabetes. Conversely, treatment of periodontal disease and reduction of oral inflammation may have positive effects on the diabetic condition, although evidence for this remains somewhat equivocal.

Key words: Adipokines; Diabetes; Periodontal disease; Periodontal therapy

Core tip: Several adipokines could serves as the monitoring molecules that reflect overall and oral disease conditions include periodontitis. Because they are rapidly change upon the change in body and oral conditions. The treatment response and disease activity progression may also predicted using these kinds of molecules. Moreover, the method to collect and analyse adipokines is relatively simple because they can be detected in gingival crevicular fluid and analysed using general enzyme-linked immunosorbent assay technology. Collectively, clinicians include medical doctors and periodontists should take the concern regarding adipokines into their routine periodontal treatment plan and management.

Original sources: Ogawa H, Damrongrungruang T, Hori S, Nouno K, Minagawa K, Sato M, Miyazaki H. Effect of periodontal treatment on adipokines in type 2 diabetes. *World J Diabetes* 2014; 5(6): 924-931 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/924.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.924

OVERVIEW OF PERIODONTITIS AND INFLAMMATION IN TYPE 2 DIABETES

Periodontal disease refers to the processes of destruction of the peri-tooth structures that support the teeth. These comprise the gingiva, the periodontal ligament, the cementum and the alveolar bone. The chronic destruction of these supporting tissues leads to the eventual loss of teeth. Epidemiological studies have revealed that more than two-thirds of the world's population suffers from

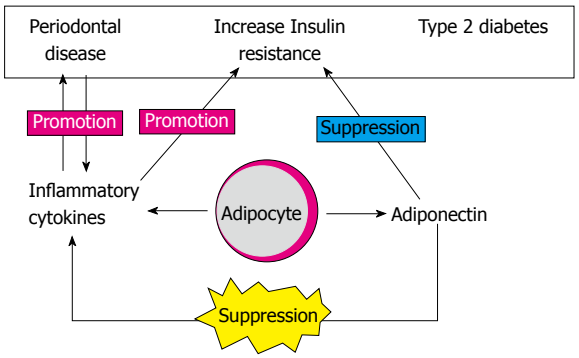

Figure 1 Relationship between type 2 diabetes and periodontal disease (hypothesis).

one of the chronic forms of periodontal disease[1].

Periodontal destruction is host-mediated by locally produced pro-inflammatory cytokines in response to the bacterial flora and its products[2]. It is possible that the production of local cytokines[3] and or low-level asymptomatic bacteremia or endotoxemia[4] affects the plasma concentration of pro-inflammatory biomarkers.

Significant differences in the plasma concentrations of such biomarkers have been described[5-8]. Periodontitis may have an even greater influence on the systemic inflammatory condition in individuals with diabetes. Elevated circulating levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and high-sensitivity C-reactive protein, which can worsen insulin resistance and thereby impair glycemic control, have been shown in several studies[9,10]. Thus, periodontal disease may have a significant impact on the metabolic state in diabetes[11]. TNF-α has been reported to play a key role in the pathogenesis of type 2 diabetes, and the correlation of this cytokine with insulin resistance has also been shown in metabolic syndrome[12].

Several studies have reported the effects of periodontal treatment on glycemic control as well as systemic inflammatory mediator levels in patients with type 2 diabetes. In some cases, positive effects such as improving HbA1c or serum level of adiponectin have been indicated[13,14]; however, such phenomena regarding adipokines are still unclear due to several confounding factors. Adipokines are molecules mainly produced and exocytosed from adipocytes. These molecules are a large family composed of members such as leptin, adiponectin, resistin, visfatin, adipsin, interleukin, monocyte chemotactic protein-I and retinol-binding protein.

Accordingly, this review focuses on providing a concise summary and dealing with recent advances regarding the potential of selected adipokines as therapeutic tools or targets of periodontal treatment (Figure 1).

ADIPOKINE MOLECULES AND PERIODONTAL TREATMENT

Leptin

Leptin, a molecule that acts as an obesity-regulatory hormone, has the cytogenetic location of 7q32.1[15]. The gene encoding leptin is named the *LEP* gene or the obese gene, which produces a 16-kDa protein secreted by white adipose tissue. By interaction with leptin receptor[16], it leads to appetite regulation, control of body energy expenditure and maintenance of bone mass. The actions of leptin mainly occur in the hypothalamus[17]; however, the production of leptin has also been found in bone marrow, placenta, skeletal muscle and stomach[17-20]. Recently, it has been found that leptin could reduce adipose tissue inflammation *via* activation of the macrophage histone deacetylase HDAC4[21]. In an animal model, namely, mice without the *LEP* gene, which are dramatically obese, leptin injection led to weight loss due to food intake reduction and increased energy expenditure[16,22].

The relationship between leptin and insulin is still not well established. At present, it has been demonstrated that leptin suppresses insulin production *via* a negative feedback loop, but insulin stimulates the production of leptin[23,24]. These interplays occur in an axis named the adipo-insular axis, and progression of insulin resistance was shown to be correlated with dysregulation of this axis[25]. Recent evidence in an *in vitro* model has demonstrated that leptin influenced insulin by regulation of insulin-like growth factor-binding protein 2[26], and this regulation occurred through signal transducers and activators of transcription (STATs), especially STAT-3, as well as phosphatidylinositol-3-kinase and the Akt signaling pathway[26,27].

Leptin and periodontal treatment

Inflammation of periodontal tissue results in an increased serum leptin level, but leptin significantly decreased ($P < 0.05$) during a 3-mo follow-up period in type 2 diabetic patients who received non-surgical periodontal treatment[28]. Even though this study and a study by Teres *et al*[29] found that leptin correlates with inflammatory condition because they found a positive relationship between IL-6 and leptin but a negative relationship between vitamin D and IL-6, the latter study failed to show that periodontal therapy could change the level of leptin as well as those of other adipokines in serum. Recent evidence has also suggested that the combination of periodontal treatment with periodontal antibiotic treatment could improve the periodontal status of Japanese type 2 diabetic patients without dramatically affecting the serum leptin level[30]. From all of the above studies, it seems that leptin is not a sensitive marker for periodontal tissue change or improvement. This molecule may reflect the systemic inflammatory conditions rather than local ones.

Adiponectin

Adiponectin (also known as Acrp30, apM1 or GBP28) is a 3-kDa adipokine secreted mainly by adipocytes, which plays important roles in the homeostasis control of glucose, energy and lipid metabolism. The adiponectin gene (*Adipoq*) is located on chromosome 3 at 3q27[31]. Although this protein is secreted mainly by adipocytes, it is also

secreted by other cell types include cardiomyocytes[32,33]. Unlike other adipokines, adiponectin exerts anti-inflammatory, anti-diabetic as well as anti-arthrogenic activities[34-36]. Attempts have been made to utilize this molecule as a therapeutic agent or for obese patients. Adiponectin exerts its activity *via* two types of receptor, namely, adiponectin receptor 1 (ADIPOR1) and ADIPOR2[37]. Both of these are widely expressed in diverse cell types, include cardiovascular and immune cells. ADIPOR1 is expressed markedly in skeletal muscle cells, whereas ADIPOR2 is expressed mainly in liver cells[37,38]. When adiponectin binds to its receptor, the signaling pathway *via* activation of peroxisome-proliferator-activated receptor-γ, AMP-activated protein kinase (AMPK) or p38 mitogen-activated protein kinase (MAPK) has been shown to be active[27]. Among these, AMPK acts as a major downstream molecule of the adiponectin signaling pathway[39].

Chronic low-grade inflammation and oxidative stress in obesity have been shown to downregulate *Adipoq* gene and protein expression[40]. TNF-α and IL-6, two main inflammatory molecules, are capable of downregulation of adiponectin *via* protein kinase C[41] and MAPK signaling[42], respectively. Moreover, adiponectin inhibits monocyte adhesion to endothelial cells as well as inhibiting macrophage function, collectively contributing to inflammatory cascade regulation[43]. In addition, adiponectin was shown to significantly induce anti-inflammatory cytokines ($P < 0.05$), for instance, IL-10 and IL-1 receptor antagonist, in human monocytes and macrophages[44]. Recently, it was also found that adiponectin could induce the pro-inflammatory function of isolated CD4+ T cells and macrophages by enhancing T-cell differentiation and the induction of interferon gamma production[45]. This suggests a new role of adiponectin in the induction of selected inflammatory stimulation for desensitizing these cells to further stimuli.

In liver, adiponectin reduces gluconeogenesis in concert with insulin and improves insulin sensitivity[46,47]. The plasma level of adiponectin in isolated human subjects is also inversely related to fasting insulin level ($r = -0.63$) and insulin resistance ($r = -0.38$)[48]. From these lines of evidence, adiponectin has been studied for the possibility of using it as a target for diabetic drugs, especially in type 2 diabetes, and also in cardiovascular diseases.

Adiponectin and periodontal treatment

In elderly patients with chronic periodontitis, serum adiponectin level is similar to that in periodontally healthy subjects, but females have a higher serum adiponectin level than males[49]. In addition, non-surgical periodontal treatment given to adult patients with mild to moderate periodontitis did not affect the serum adiponectin level[29]. This may be explained by the fact that adiponectin has different isoforms (low, middle and high molecular weight)[50] with different functions. In addition, it was suggested that only the ratio of high-molecular-weight adiponectin to total adiponectin was significantly lower in subjects with periodontitis[51]. Furthermore, diabetic patients with periodontitis who received periodontal treatment without or with topical antibiotics showed significant elevation of serum adiponectin compared with an untreated group ($P < 0.05$)[28,30]. Effective control of inflammation by periodontal treatment with local antibiotics may contribute to increase systemic anti-inflammatory markers such as adiponectin and hence improve overall health status[14].

Resistin

Resistin [also known as adipocyte-specific secretory factor and found in inflammatory zone (FIZZ)] is a 12.5-kDa protein said to play a role as a mediator of insulin resistance[52]. The name resistin comes from the finding that this molecule provides resistance to insulin. The gene that encodes this molecule, named *Retn*, is located on chromosome 19 at p13.3[53]. Interestingly, in humans, resistin is predominantly secreted by macrophages, rather than adipocytes[54]. Bone marrow, peripheral mononuclear cells, lung[55], placenta tissue[56] and pancreatic β-cells[57] can also express this molecule. Murine adipocytes, when cultured in the presence of insulin-sensitizing drugs, for example, thiazolidinediones, appeared to exhibit suppressed resistin secretion[53]. Circulating resistin was shown to decrease upon the administration of anti-diabetic drugs such as rosiglitazone, and to be increased in diet-induced and genetic forms of obesity. From these lines of evidence, it has been postulated that resistin may function as a link between obesity and diabetes, especially type 2 diabetes. However, one study did not find any relationship between resistin and obesity or insulin resistance[54]. This controversial finding may be explained in part by the fact that resistin has at least 2 isoforms: a high-molecular-weight hexamer form and a more bioactive but less prevalent low-molecular-weight trimer form, which exerts a different biological function[27,58]. Numerous clinical studies have demonstrated a possible relationship of resistin and insulin resistance in obese people with or without diabetes. The possible contributing factor that links resistin to insulin resistance may be hyperresistinemia. In addition, recent clinical studies have shown that individuals with a high serum resistin level have a significantly increased risk of developing type 2 diabetes[59,60].

Resistin may play a pivotal role in monocyte-macrophage function and inflammation due to the finding that the expression of resistin was increased in concert with the maturation of monocytes into macrophages[55]. At present, the concrete mechanism of resistin-mediated inflammation has not yet been established due to the resistin receptor not being identified yet, but an isoform of decorin and tyrosine kinase-like orphan receptor 1 were proposed as functional resistin receptors that may modulate glucose homeostasis or regulate enlargement of white adipose tissue in rodents[61,62]. Many pro-inflammatory stimuli and cytokines including lipopolysaccharide, TNF-α, IL-6 and IL-1β are capable of inducing resistin expression and function[63-65]. One line of evidence suggested that resistin could also induce the secretion of pro-inflammatory cyto-

kines, for instance, TNF-α, IL-6, IL-12 or monocyte chemoattractant protein-1 in peripheral blood mononuclear cells and macrophages[65,66]. Collectively, these findings show that resistin is a molecule that is closely related to systemic inflammation.

Resistin and periodontal treatment

The relationship between serum resistin and periodontal condition was investigated by Furugen *et al*[49], who found that serum resistin and total leukocyte count in subjects with periodontitis were higher than those in subjects without 6-mm pocket depth or without bleeding on probing, with an odds ratio of 2.0 or more. Saito *et al*[67] also found an association between increased severity of periodontitis and increased serum resistin level both in bivariate (OR = 3.0; 95%CI: 1.2-7.6) and multivariate analyses (adjusted OR = 3.1; 95%CI: 1.1-8.6) analyses, and concluded that the increased levels of serum resistin in middle-aged women might affect their systemic health. After non-surgical periodontal treatment, the serum resistin level in periodontitis patients who have no underlying disease decreased to some extent[68]. Recently, periodontal treatment with antibiotics in type 2 diabetic patients was shown to result in no difference of serum resistin level compared to that of healthy counterparts[30]. However, this study was performed in only a small number of subjects (21 subjects) and all subjects were categorized into mild periodontitis. The effect of periodontal treatment on serum resistin needs to be more clearly elucidated in a larger sample.

Visfatin

Visfatin, a 52-kDa protein, is another adipokine secreted by adipocytes and mimics the effect of insulin[69]. This molecule was found to be enriched in visceral adipose tissue, which is the reason for its name. It was also known as pre-B-cell colony-enhancing factor (PBEF)[27] or nicotinamide phosphoribosyltransferase (Nampt)[70] PBEF or Nampt, with the gene located on chromosome 7 at q22.3[71]. Visfatin is essential for nicotinamide adenine dinucleotide biosynthesis and hence is related to cell metabolism. In humans, visfatin is mainly expressed in bone marrow (highest expression in leukocytes), liver and muscle cells. It is also expressed in various tissues, including heart, lung, kidney and placenta. Visfatin has 2 isoforms: intracellular and extracellular ones. The intracellular isoform mainly functions in energy production in cells, while the extracellular isoform is related to increased inflammatory cytokines, such as TNF-α, IL-1β, IL-16 and transforming growth factor-β1, and the chemokine receptor C-C chemokine receptor type 3[72].

Visfatin has insulin-mimicking effects, for example, increasing glucose uptake and enhancing triglyceride biosynthesis, because it binds to the insulin receptor, although at a different site from insulin[69]. In type 2 diabetic individuals, it was demonstrated that visfatin impaired vascular endothelial function as well as creatinine clearance[73], which probably leads to atherosclerosis and chronic kidney disease. Additionally, the visfatin level in this type of patient was found to be enhanced, which positively correlated with increased homocysteine, an endothelial dysfunction marker[74]. It seems that visfatin levels are positively associated with a series of inflammatory conditions, independently of other potential metabolic implications[75].

Research has mainly focused on the role of visfatin in cardiovascular diseases. As mentioned earlier, it was shown to induce inflammation of endothelial cells and vascular smooth muscle cells. It also induced TNF-α and IL-8 production from peripheral mononuclear cells[76]. Additionally, macrophage survival was promoted by visfatin[77]. Exogenous visfatin could stimulate inducible nitric oxide synthase, which is a pro-inflammatory cytokine that contributes to endothelial dysfunction and vascular injury in diabetes-related vascular complications[78,79].

Visfatin and periodontal treatment

Because visfatin exerts pro-inflammatory functions in several organs, this molecule also correlates with chronic inflammation of periodontal tissue. In periodontitis, it was reported that visfatin concentration was increased in such patients and the more severe the periodontitis, the higher the level of visfatin observed in serum and gingival crevicular fluid (GCF)[80]. Another study was performed on an observational basis in healthy subjects, those with periodontitis without diabetes and those with periodontitis with diabetes; it was found that the mean visfatin in both serum and GCF was markedly increased in diabetic patients concurrently burdened by periodontitis[81]. The periodontal ligament cells could produce visfatin and *Fusobacterium nucleatum*, one of the periodontopathic bacteria, enhanced the level of visfatin, which supports the assertion that bacteria exert an inflammatory bioburden on periodontal tissue. This effect could be reversed by biomechanical loading[82]. The effect of non-surgical periodontal treatment on serum and GCF visfatin level in periodontitis patients was reported by Raghavendra *et al*[83], who found that periodontal treatment given to periodontitis patients could decrease a high visfatin level in the active disease stage to a nearly normal level, as in periodontally healthy individuals both GCF ($P < 0.001$) and serum ($P = 0.008$). Although no study has yet been conducted on the effect of non-surgical periodontal treatment on the level of visfatin in periodontitis patient with diabetes, it seems that this molecule is associated with inflammatory conditions and can be used as an inflammatory marker or periodontal disease activity marker at both local and systemic levels.

Adipsin

Adipsin, also known as complement factor D, factor D and adipocyte trypsin, is one of the adipokines secreted by adipocytes into the bloodstream. The adipsin gene in humans is located at p13.3 on chromosome 19[84]. Adipsin belongs to the serine protease family and functions in cleavage of the bond between complement factor 3

and factor B[85]. Human adipsin is a 24-kDa molecule that stimulates acylation-stimulating protein and is then involved in the stimulation of glucose transport, enhancement of fatty acid re-esterification and facilitation of lipid lipolysis[86]. In humans, plasma levels of adipsin are not different or slightly increased in the obese population compared with the non-obese one[87,88], but this remains controversial. Recently, it has been demonstrated *in vitro* that high glucose promoted adipocyte-derived molecules including adipsin and resistin, but inhibited osteogenic differentiation in osteosarcoma (MG-63) cells[89]. Recently, adipsin level was increased and positively correlated with lung fibrosis ($r = 0.412$, $P < 0.001$) and pleural plaque ($r = 0.245$, $P = 0.043$), in asbestos-exposed workers[90]. This suggested the role of adipsin in inflammation enhancement.

Adipsin and periodontal treatment

Concerning the role of adipsin in periodontitis, it was suggested that it exerted the same activity as *P. gingivalis*, resulting in the breakdown of periodontium[91]. The effect of periodontal treatment on the change of adipsin in human subjects has not been reported yet, but we hypothesize that this molecule might be decreased as a result of inflammatory reduction after periodontal therapy.

PERSPECTIVES

Adipokines are much more complex and involved in many systems, include immune and endocrine systems, and these molecules influence the pathogenesis of obesity-related diseases, particularly type 2 diabetes and cardiovascular diseases, as well as inflammatory diseases, especially periodontitis. A growing number of molecules have been identified to be secreted from adipocytes and more are yet to be discovered. Unravelling their orchestrated roles in controlling obesity, inflammation and periodontal health may lead to successful management of pathological conditions. Some markers, especially visfatin, are molecules that are closely related to inflammation, diabetic condition and periodontitis. With the recent development of sophisticated means to study molecules, we now aim to detect, analyze and make use of a number of molecules simultaneously to screen, explain and monitor the therapeutic outcome of disease conditions. This is due to no single molecule being able to reflect the nature of complex multifactorial diseases such as periodontitis and diabetes. Thus, the disease profile should be set as a template from several integrated adipokines, not only quantitatively for each molecule but also qualitatively. Here, single-nucleotide polymorphisms of each gene controlling these adipokines should be taken into account for periodontitis staging in diabetic patients and evaluating the disease response.

Not only data from serum but also data from non-invasive methods, for instance, analyses of gingival crevicular fluid and saliva, should be utilized as robust confirmation of local periodontal health. An ideal marker for periodontitis will not only demonstrate a clear relationship with periodontitis, but also be linked to systemic conditions that are influenced by periodontitis. To develop an adipokine candidate to use as a periodontal disease-specific biomarker or therapeutic compound, we also need to perform experiments mainly in human subjects to complete our understanding of the mechanism of such substances.

Robotic science has emerged as an important field in medicine. In the next century, *in vitro* robot-assisted synthesis of therapeutic molecules that combines the advantages of each adipokine will probably be launched on the market and make a major contribution to the treatment of severe periodontal breakdown, more effectively than contemporary therapeutic modalities. At that time, periodontitis in diabetic patients may no longer be a major oral health problem.

CONCLUSION

Current knowledge concerning the roles of major adipokines provides only a partial understanding of their associations with the pathogenesis of periodontitis in type 2 diabetes. This is probably due in part to the limited number of studies conducted on an acceptable number of human subjects. More studies regarding the effect of periodontal therapy on several adipokines should be performed. Nevertheless, we saw potential to develop visfatin as a tool for drug discovery and to generate more specific therapeutic targets. A novel cocktail of adipokine-related therapeutic strategies may offer opportunities for the successful management of periodontitis concomitant with diabetes.

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P- Reviewer: Hassan M, Kucherlapati MH
S- Editor: Tian YL **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength?

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Received: November 27, 2013 Revised: January 26, 2014
Accepted: June 20, 2014
Published online: August 15, 2014

Abstract

Diabetes mellitus is a chronic condition that occurs when the body cannot produce enough or effectively use of insulin. Compared with individuals without diabetes, patients with type 2 diabetes mellitus have a considerably higher risk of cardiovascular morbidity and mortality, and are disproportionately affected by cardiovascular disease. Most of this excess risk is it associated with an augmented prevalence of well-known risk factors such as hypertension, dyslipidaemia and obesity in these patients. However the improved cardiovascular disease in type 2 diabetes mellitus patients can not be attributed solely to the higher prevalence of traditional risk factors. Therefore other non-traditional risk factors may be important in people with type 2 diabetes mellitus. Cardiovascular disease is increased in type 2 diabetes mellitus subjects due to a complex combination of various traditional and non-traditional risk factors that have an important role to play in the beginning and the evolution of atherosclerosis over its long natural history from endothelial function to clinical events. Many of these risk factors could be common history for both diabetes mellitus and cardiovascular disease, reinforcing the postulate that both disorders come independently from "common soil". The objective of this review is to highlight the weight of traditional and non-traditional risk factors for cardiovascular disease in the setting of type 2 diabetes mellitus and discuss their position in the pathogenesis of the excess cardiovascular disease mortality and morbidity in these patients.

Key words: Type 2 diabetes mellitus; Cardiovascular disease; Dyslipidaemia; Blood pressure; Obesity; Microalbuminuria; Inflammation; Insulin resistance; Postprandial Hyperglycaemia; Homocysteine

Core tip: The objective of this review is to highlight the importance of traditional and non-traditional risk factors for cardiovascular disease in the setting of type 2 diabetes mellitus and discuss their position in the pathogenesis of the excess cardiovascular disease mortality and morbidity in these patients.

Original sources: Martín-Timón I, Sevillano-Collantes C, Segura-Galindo A, del Cañizo-Gómez FJ. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World J Diabetes* 2014; 5(4): 444-470 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i4/444.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i4.444

INTRODUCTION

Diabetes mellitus (DM) is a chronic condition that occurs when the body cannot produce enough or effectively use of insulin, and are induced by a genetic predisposition coupled with environmental factors[1].

Three hundred sixty six million people have DM

in 2011; half of these (183 million people) are undiagnosed[2]. The number of people with DM worldwide is increasing and by 2030 this will have risen to 552 million[2].

DM is a well-established risk factor for cardiovascular disease (CVD). People with type 2 diabetes mellitus (T2DM) have a higher cardiovascular morbidity and mortality, and are disproportionately affected by CVD compared with non-diabetic subjects[3]. Diabetic vascular disease is responsible for two-four-fold rise in the occurrence of coronary artery disease (CAD) and stroke, and two-eight-fold improve in the risk of heart failure[4]. It has been described that patients with T2DM and no previous history of CAD have the similar risk for cardiac events as subjects with a prior myocardial infarction[5]. However, subsequent studies have revealed variable results[6], which more indication that diabetes status may not be a CVD equivalent in all conditions, thus highlighting the necessity for multivariate approach as an suitable basis for risk stratification for CVD prevention in persons with diabetes[7]. The CVD risk follows a gradient, and taking this gradient depends on the combination of numerous risk factors[7]. Most of this excess risk is it associated with an improved prevalence of well-known risk factors such as hypertension, dyslipidaemia and obesity in these subjects. During the recent decade, conclusive evidence has been gathered that treatment of traditional risk factors is of immense importance for patients with T2DM in the reduction of CVD risk[8,9]. The poor control of the majority of cardiovascular risk factors observed in the diabetic population[10] supports the need for more aggressive arrangement of modifiable cardiovascular risk factors, especially in patients with previous CVD. However the improved cardiovascular disease in T2DM patients cannot be attributed solely to the higher prevalence of traditional risk factors. Therefore other non-traditional risk factors may be important in people with T2DM[11] (Table 1). Very few studies have shown prospectively the association of non-traditional risk factors in T2DM, independent of traditional risk factors[12]. Moreover therapies that are currently used in the management of T2DM such insulin-sensitizers and statins have a variety of effects on many of these non-traditional risk factors[13,14]. The relative magnitude of these risk factors has been widely reviewed in the literature[15].

Several studies have aided elucidate the mechanisms underlying the vascular dysfunction that leads to cardiovascular outcomes in DM. This vascular dysfunction is related with visceral adiposity, insulin resistance (IR) and changes in the levels of a diversity of circulating factors[16]. The atherogenesis begins as an endothelial cell dysfunction when various noxious insults as dyslipidaemia, hypertension, diabetes, smoking, *etc.* induce deficits of nitric oxide (NO) and prostacyclin. Next, mononuclear cells such as monocytes and T lymphocytes binding to the endothelium; this process is mediated by adhesion molecules present on the endothelial surface, such as vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and E-selectin. Monocyte migrates into the sub endothelial space, matures into a resident macrophage and takes up lipid through certain scavenger receptors such as SR-A and CD-36, becomes a foam cell. Later, smooth muscle cells migrate to the surface and form the fibrous cap of the lesion, and lastly lipid-laden macrophages release matrix metalloproteinase' s causing plaque rupture and acute coronary syndromes such as myocardial infarction and unstable angina. Oxidative stress (OE) play an important role in atherogenesis, especially in DM[17,18], by proatherogenic role of oxidized low-density lipoprotein and its "*in vivo*" existence[19,20]. Elements that may promote increased OE in DM comprise antioxidant deficiencies, increased production of reactive oxygen species and the process of glycation and glycooxilation[20]. Increased plasma levels of nitrotyrosine, a marker of protein oxidation[21,22], elevated both plasma and urine levels of F2-isoprostane, a marker of OE[21-23] also the evidence of oxidative damage to DNA[24], was observed in patients with T2DM.

Table 1 Cardiovascular risk factors in diabetes mellitus

Traditional	Nontraditional
Dyslipidaemia	Insulin resistance and Hyperinsulinemia
Hypertension	Postprandial Hyperglycaemia
Obesity	Glucose variability
Abdominal obesity	Microalbuminuria
Physical exercise	Haematological factors
Cigarette smoking	Thrombogenic factors
	Inflammation C-reactive protein
	Homocysteine and vitamins
	Erectile dysfunction
	Genetics and Epigenetics

In summary, CVD is elevated in T2DM due to a complex combination of various traditional and non-traditional risk factors, that have an important role to play in the beginning and the evolution of atherosclerosis over its long natural history from endothelial function to clinical events[25]. The clustering of vascular risk observed in association with IR has led to the view that cardiovascular risk appears early, before the development of T2DM, whereas the solid interactions between hyperglycaemia and microvascular disease suggests that this risk is not appear until frank hyperglycaemia appears. These notions highlight the progressive nature of both T2DM and related cardiovascular risk which propose specific challenges at diverse stages of the life of a subject with DM[26]; but do diabetic patients have specific risk factors which could explain the observed increase in CVD, or have all cardiovascular risk factors, traditional and non-traditional, the same strength?

The objective of this review is to highlight the weight of traditional and non-traditional risk factors for CVD in the setting of T2DM and debate their position in the pathogenesis of the excess CVD mortality and morbidity in these patients. It is essential to know that these risk factors do not act in isolation. Risk factors occur simultaneously[27], compounding the risk for a cardiovascular event, although such interactions are difficult to quantify

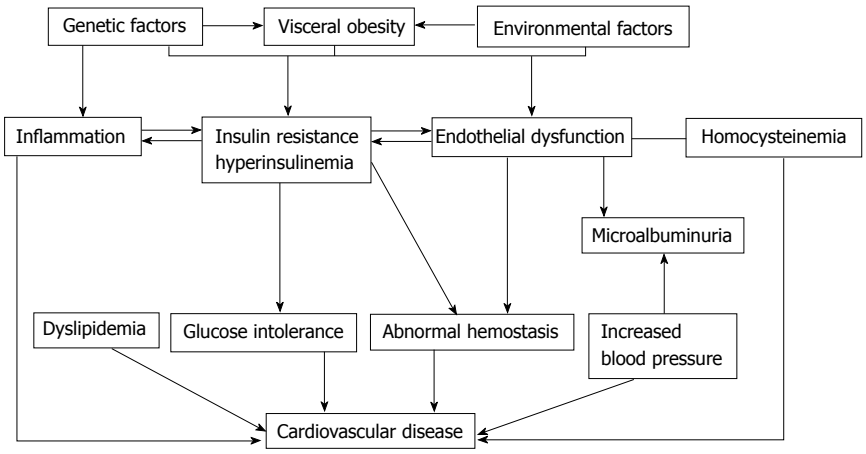

Figure 1 Interactions of traditional and non-traditional risk factors in diabetes mellitus.

(Figure 1). Many of these risk factors may be common history for both DM and CVD, reinforcing the postulate that both disorders come independently from "common soil"[28].

TRADITIONAL RISK FACTORS

Dyslipidaemia

In T2DM, IR increases the mobilization of free fatty acids from adipose tissue. There are three mechanisms across which there is increased very low-density lipoproteins hepatic production: an increased lipogenesis, an exacerbation of substrate availability, and decreased apolipoprotein B-100 (ApoB) degradation. These changes carry to a lipid profile marked by low high-density lipoprotein cholesterol (HDL-C), high triglycerides (TGs), increased ApoB synthesis and small dense LDL particles[29]. This LDL subtype is more inclined to oxidation, playing an important role in atherogenesis. Stronger than LDL cholesterol, a low HDL-C or lonely elevated TGs, atherogenic dyslipidaemia (Low HDL-C and ApoA, Elevation of both fasting and post-prandial TGs, Elevation of small dense LDL particles, Elevation of ApoB) is in T2DM patients a self-determining predictor of cardiovascular risk. The protective function of HDL may be lost in type 2 diabetics owing to alterations of the protein, resulting in a pro-oxidant, inflammatory phenotype[30].

Association between dyslipidaemia and cardiovascular risk in T2DM: A causal association exists between elevation of TGs-rich particles and their remnants, low HDL-C and cardiovascular risk[31,32] as is shown in large data from case-control, genetic, and large observational studies. Still in patients with a normal LDL-C levels, results from statin trials confirm the place of low HDL as an independent cardiovascular risk marker[33,34]. Cardiovascular event rates were significantly greater in those with dyslipidaemia: LDL-C > 2.6 mmol/L, HDL-C $\leqslant$ 0.88 mmol/L and TGs $\geqslant$ 2.3 mmol/L[35,36], as is proved in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study and in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study. The FIELD study[37] defined the following variables as best predictors of cardiovascular events during a five year monitoring: lipid ratios non-HDL/HDL-C and total/HDL-C. Ratio of ApoB/ApoA is also associated to CVD outcomes, but this ratio wasn't superior to conventional lipid ratios. Data from the Emerging Risk Factor Collaboration (ERFC) study[38] with 302430 persons with no history of cardiovascular disease, demonstrated that Apo B and non-HDL-C each had very similar association with coronary heart disease (CHD) regardless of the existence of diabetes. The ERFC study showed that an increase of 0.38 mmol/L or 15 mg/dL in HDL-C was associated with a 22% reduction in risk of CHD. Non-HDL-C was the best tool to define the risk linked with TGs rise in clinical practice[38].

Management of dyslipidaemia, significance in the prevention of CVD in T2DM: As the development of atherogenic dyslipidaemia precedes the onset of overt glycaemia and the clinical diagnosis of diabetes, early effective intervention is recommended to reduce the risk of premature CVD.

In T2DM large data exists on action mechanism and efficacy of statins in the prevention of CVD events[39]. The Collaborative Atorvastatin Diabetes Study assessed the benefits of a statin in T2DM patients and at least one of the following risk factors: albuminuria, retinopathy, hypertension or current smoking[40]. In this study, 2838 type 2 diabetics were randomized to placebo or atorvastatin 10 mg/d. The study was finished ahead of time, because to a 37% reduction (P = 0.0001) in the primary endpoint (first acute CHD event). In the Heart Protection Study, simvastatin (40 mg/d) reduced the composite primary endpoint by 33% (P = 0.0003)[41]. This study was performed with 2912 patients (mainly T2DM) without pre-existing CVD. Also, atorvastatin 10 mg decreased the rate of major CVD events in 23% in the Anglo-Scandinavian Cardiac Outcomes Trial subgroup. Diabetic patients were free from CVD[42].

Residual risk in people on LDL-lowering therapy: Patients with T2DM at the LDL-C target are still at a

Table 2 Recommendations for blood pressure control in diabetes

Recommendations	Class	Level
Blood pressure control is recommended in patients with diabetes mellitus and hypertension to lower the risk of cardiovascular events	I	A
It is recommended that a patient with hypertension and diabetes mellitus is treated in an individualized manner, targeting a blood pressure of < 140/85 mmHg	I	A
It is recommended that a combination of blood pressure lowering agents is used to achieve blood pressure control	I	A
A RAAS blocker (ACE-I or ARB) is recommended in the treatment of hypertension in diabetes mellitus, particularly in the presence of proteinuria or microalbuminuria	I	A
Simultaneous administration of two RAAS blockers should be avoided in patients with diabetes mellitus	III	B

ACE-I: Angiotensin converting enzyme-inhibitors; ARB: Angiotensin receptor blockers; RAAS: Renin angiotensin aldosterone system; Class: Class of recommendation; Level: Level of evidence.

significant risk of CVD events[31]. This residual risk is associated to several factors as increased on TGs-rich proteins, decreased HDL-C and small, dense LDL particles. Data of FIELD study demonstrated that fenofibrate therapy did not decrease the primary endpoint (non-fatal myocardial infarction and CAD-related death), but total CVD events were decreased from 14% to 12.5% (P = 0.035)[35,43]. However, a subgroup analysis of dyslipidaemic people (TGs > 2.3 mmol/L and HDL-C ≤ 0.9 mmol/L) in this study showed a 27% reduction in CVD risk[35]. In the ACCORD trial, 5518 patients were allocated to fenofibrate plus simvastatin (20-40 mg daily) or placebo without any additional effect on the primary endpoint. In a pre-specified subgroup analysis of people with TGs > 204 mg/dL and HDL-C < 34 mg/dL, cardiovascular risk was decreased in 31% in the fenofibrate-plus-simvastatin group[44]. In both ACCORD and FIELD, treatment with fenofibrate was related with a strong reduction of TGs (22%), whereas increase of HDL-C remained less than expected (2% and 2.4%, respectively). The clinical benefits of fibrates on major CVD events have been confirmed in meta analyses; but not on cardiovascular mortality[43,44]. The effects seem to be appeared to an improvement in TGs[45].

Blood pressure

Arterial hypertension is present in more than 60% of T2DM patients[46]. This is directly linked to: (1) increased renin-angiotensin-aldosterone system activity; (2) hyperinsulinemia associated to increased renal reabsorption of sodium; and (3) increased sympathetic tone[47]. Aging, obesity, and the onset of renal disease also promote an increase in the prevalence of hypertension. Hypertension and DM are additive risk factors for CVD. While the diagnosis of diabetes doubles the cardiovascular risk in men and more than triples the risk in women, hypertension quadruple cardiovascular risk in diabetic patients[5,48].

Treatment targets: Lowering blood pressure (BP) under 140 mmHg systolic and 85 mmHg diastolic (Table 2) have shown positive effects on cardiovascular outcomes in randomized controlled trials[49-52]. The United Kingdom Diabetes Prospective Study (UKPDS) showed that strict (mean 144/82 mmHg), compared with less strict (mean 154/87 mmHg) control decreased macrovascular events by 24%. DM-related mortality decreased 15% with each 10 mmHg drop, down to a systolic BP (SBP) of 120 mmHg, with no indication of further decrease, as it was shown in a post-hoc observational analysis of the UKPDS trial[53]. Later, the ACCORD trial doesn't support a decrease of SBP below 130 mmHg[50].

Recent evidence suggests visit-to-visit variability in SBP and masked hypertension are predictors of cardiovascular disease in T2DM.

Effects of visit-to-visit variability in SBP on CVD in T2DM patients[54]: Using the data from ambulatory BP monitoring, previous studies reported that short-term or circadian variability of BP was an important prognostic factor of cardiovascular outcomes[55-58]. Similarly, a number of observational studies have investigates the impact of long-term or visit-to-visit BP variability on the risks of cardiovascular outcomes[59-63]. In the Blood Pressure-Lowering Arm of the Anglo-Scandinavian Cardiac Outcomes Trial, Rothwell *et al*[59] reported that visit-to-visit SBP variability was a strong predictor of CVD among patients with transient ischemic attack or stroke and among hypertensive patients. In the Action in Diabetes and Vascular Disease (ADVANCE) Trial, which included 8811 patients, visit-to-visit SBP variability was clearly associated with myocardial infarction and cardiovascular death. Another new and important finding of this analysis was that visit-to-visit variability of SBP clearly predicted the future development of major microvascular complications among patients with T2DM[54].

Risk associated with masked hypertension in T2DM patients[64]: Masked hypertension (MH) is defined as an ambulatory hypertension with a normal conventional BP (CBP).

The International Database on Ambulatory BP (ABP) in relation to Cardiovascular Outcomes[65], which contain a great number of diabetic patients, many of whom have MH, detected a higher prevalence of MH in DM than in non DM, and this finding was even more remarkable in treated *vs* non treated diabetics. Currently is not known the mechanism by which antihypertensive treatment is linked with a higher prevalence of MH. Cardiovascular risk in diabetic patients who are not receiving antihypertensive treatment and presenting with MH was significantly higher than in their normotensive comparator group. In contrast, antihypertensive-treated diabetics with MH had cardiovascular risk that was identical to treated stage 1 and stage 2 hypertensive subjects. This suggests that a significant percentage of these subjects had real hypertension that simulated MH in the presence of elevated

ABP and normalized CBP[65].

Nevertheless, currently, there aren't credible studies in diabetics with MH to evidence the benefit of antihypertensive therapy or to indicate how low to go with the reduction in ABP to achieve optimal reduction in cardiovascular risk. We may have to balance the potential advantage of further reduction in systolic ABP and CBP values with the increased cardiovascular risk of lower diastolic ABP and CBP.

Obesity and abdominal obesity

Generalised obesity assessed by the body mass index (BMI), and abdominal obesity determined by the waist circumference (WC), are related with a variety of CVD risk factors. Clinical guidelines do not indicate whether BMI or the WC measurements have identical utility in predicting cardiovascular risk in individuals with T2DM compared to non-diabetic patients[66,67].

The impact of obesity on both atherogenesis and in novel procoagulant and prothrombotic cardiovascular risk factors is of particular interest in cases of T2DM, as they contribute to increased CVD mortality in these individuals[68-72].

In diabetic patients the coexistence of multiple variables such as diabetic duration, glycaemic control and the drugs used for achieving it, lipid profile, BP or the existence of risk behaviours such as smoking or alcohol use may confound the impact of obesity on the risk of CVD[73].

The Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) study[73] was designed to establish the association between indexes of obesity and atherothrombotic risk factors in patients with T2DM and document CVD. By only taking into account this study's baseline it was possible to evaluate among this group of patients if a higher BMI or higher WC was associated with specific cardiovascular risk factors, and whether a higher WC was related with cardiovascular risk factors independent of diabetic patient's BMI. The review of the study baseline results showed, on the one hand, that patients with BMI $\geqslant$ 40 experienced more cases of heart failure. However, a history of myocardial infarction was less common in patients with BMI $\geqslant$ 35 (26%-30%) than in those with BMI $\leqslant$ 29.9 (34%-36%), possibly because patients with BMI $\geqslant$ 35 reported fewer years smoking than those with BMI of $\leqslant$ 29.9. Smoking was proportionally, inversely related to BMI. Furthermore the BMI, independent of the WC, had a strong association with SBP, the plasminogen activation inhibitor type 1 (PAI-1), the C-reactive protein (CRP) and fibrinogen, whereas WC had robust associations with the HDL-C and TGs levels.

It is well known that CVD is among the most frequent causes of mortality for diabetics and obese individuals. Studies have established the mortality risk in obese T2DM subjects taking the age into account. The data obtained from a study conducted in Verona with 3398 T2DM patients who were followed up for 10 years showed that in patients > 65 years a moderate excess weight predicted longer survival, whereas obesity was a negative prognostic factor in patients < 65 years[74].

On the other hand, the ADVANCE study compared the association between cardiovascular risk and BMI, WC, and the waist to hip ratio in 11140 T2DM patients, and reached the conclusion that the waist to hip ratio is the best predictor of cardiovascular events and mortality in diabetics[75].

Physical exercise

Regularly practicing physical exercise is correlated with a lower risk of cardiovascular morbidity and mortality, both in primary and in secondary prevention. However it should be taken into account that this type of evidence is often subject to other lifestyle changes that take place together with exercise (for example stopping smoking, a balanced diet, *etc.*)[76,77].

Multiple observational studies, conducted in diabetic patients, support that stated above. One such case is an American prospective cohort study of 2896 T2DM adults which showed that those who walked at least two hours per week had lower frequency of CVD mortality compared with inactive patients (HR = 0.66; 95%CI: 0.45-0.96; 1.4% *vs* 2.1% per year, respectively), and that the risk was even lower for those who walked 3 or 4 h a week. In this study the protective effect of exercise was independent of gender, age, race, BMI, diabetes duration, coexisting comorbidities and physical limitations. The authors estimated that one death per year would be prevented for every 61 individuals with diabetes who were persuaded to walk at least two hours per week[78]. The same occurred in a Finnish study, with 3316 diabetic patients, who showed that physical activity at work and during leisure time was linked with a decrease in cardiovascular mortality and total mortality[79].

It is important to note that patients with T2DM have a reduced capacity for exercise due to age, the high BMI and the frequent presence of left ventricular dysfunction[80]. Exercise improves insulin sensitivity in diabetic patients in the same way as it does in non-diabetic patients[81-83]. Patients with diabetes have greater IR which can be mediated by different defects in the glucose metabolism, and some of which would improve with physical exercise. These defects include not only a decreased number of insulin receptors and glucose transporters, but also a reduction in the intracellular enzymes activity (pyruvate dehydrogenase and glycogen synthase) and reduced oxygenation during exercise. Increased physical activity achieves higher mitochondrial enzyme activity and increases insulin sensitivity; however the number of muscle capillaries in diabetic patients with microvascular complications does not increase or is practically negligible[84-86].

Multiple studies have shown physical exercise to improve cardiovascular risk factors (dyslipidaemia, hypertension and body composition) in patients with T2DM[87]. Although it is not all kinds of physical activity exert the same influence on this risk. Aerobic exercise only or

Table 3 Suggested mechanisms for the influence of smoking on risk of type 2 diabetes

Direct effects due to inhalation of smoke from tobacco products
Impaired insulin sensitivity based on influence of haemodynamic dysregulation in capillary vascular bed
Impaired insulin sensitivity due to increase in inflammatory markers secondary to bronchitis and pulmonary infections caused by smoking
Impaired beta-cell function due to toxic effects of tobacco smoke
Lipotoxicity due to influence of increased triglyceride levels
Hypercortisolaemia and increase in abdominal fat tissue
Elevated sympathetic nervous activation
Indirect effects on glucose metabolism
Unhealthy lifestyle in smokers (poor diet, lack of physical activity)
Increased alcohol consumption (toxic effects on beta cells)
Psychosocial stress and impaired sleep associated with smoking
Impaired fetal growth in smoking pregnant women, associated with increased diabetes risk in offspring in adult life

Table 4 The strategic "five As" for smoking cessation

A-ASK:	Systematically inquire about smoking status at every opportunity
A-ADVISE:	Unequivocally urge all smokers to quit
A-ASSESS:	Determine the person's degree of addiction and readiness to quit
A-ASSIST	Agree on a smoking cessation strategy, including setting a quit date, behavioral counseling, and pharmacological support
A-ARRANGE	Arrange a schedule for follow-up

combined with resistance exercise improves glycaemic control, BP, the amount of TGs and WC. But resistance exercise alone does not have a clear impact on cardiovascular risk factors.

In prospective cohort studies, exercise was associated with improved CVD and reduced cardiovascular mortality and total mortality in patients with T2DM[88]. Results from the Nurses' Health Study[89] reported that 5125 women with T2DM who exercised for at least 4 h per week had a 40% lower risk of developing CVD (comprising heart disease and stroke) compared to those who did not. This risk improvement remained after adjustments for smoking, BMI, and another cardiovascular risk factors.

Smoking

Smoking is linked with deterioration in metabolic control in diabetic patients[90,91], which is associated with an increased risk for development of macrovascular and microvascular complications and mortality in DM[92,93].

The suggested mechanisms for the influence of smoking on risk of T2DM are summarized in Table 3. Administration of nicotine rise the circulating levels of insulin-antagonistic hormones (growth hormone, catecholamines and cortisol)[94-97], and also has been proved to affect the autonomic nervous system[98,99]. Nicotine, *via* these and possibly also other mechanisms, decreases insulin sensitivity, directly or indirectly. Also smoking increases circulating free fatty acid levels[95], and this is an additional negative factor for the insulin-mediated glucose uptake[100].

Smoking and macrovascular complications in T2DM: CVD is responsible for the main proportion of mortality associated with T2DM. There is evidence that smoking improves the risk of CAD in T2DM. Based on data from 4540 patients with T2DM followed in the UKPDS, smoking was shown to rise the risk of CHD[101] in males and females with T2DM. The expected RR incidence of a fatal or non-fatal myocardial infarction or sudden death attributable to smoking was 1.350 (95%CI: 1.11-1.59). This study reveals that smoking is an independent and significant risk factor for stroke[102] and peripheral vascular disease[15].

However, it was proved that smoking is significantly related with an augmented risk for CHD, but not for stroke, in T1 and T2DM patients in the London cohort of the prospective (8-year follow-up) World Health Organization Multinational Study of Vascular Disease in Diabetics[93].

In a prospective cohort of female nurses with T2DM[103], cigarette smoking was found to be robustly associated with the risk of CHD, and this risk improved with the number of cigarettes smoked per day. Compared with the nurses who had never smoked, the RR for CHD was 1.21 (95%CI: 0.97-1.51) for past smokers; 1.66 (95%CI: 1.10-2.52) for current smokers of up to 14 cigarettes per day; and 2.68 (95%CI: 2.07-3.48) for current smokers of 15 cigarettes per day or more.

A relatively large prospective study examined the effects of smoking cessation on cardiovascular risk in diabetic patients[104]. Data from this study reveal that stopping smoking decreases mortality risk in diabetes, but risks keep increased some years after stopping and are highly dependent on the duration of smoking.

Diabetic patients who are current smokers should be proposed a planned smoking cessation program that includes pharmacological treatment if is necessary. Detailed instruction should be provided according to the five A principles (Table 4) as is developed in the 2012 Joint European Prevention Guidelines[105].

NON-TRADITIONAL RISK FACTORS

Insulin resistance and hyperinsulinemia

IR is a principal characteristic of T2DM and it develops in multiple organs involving the skeletal muscle, liver, adipose tissue and the heart. The onset of hyperglycaemia and diabetes is often preceded by several years of IR. Obesity plays a major role in this phenomenon and provides an important link between T2DM and the accumulation of fat[106]. A significant section of the population with T2DM is obese[107].

The hyperinsulinemia, as a result of IR, occurs even before the onset of DM, and could be, by chance, related to vascular disease[108-111].

The IR, measured by the hyperinsulinaemic-euglycemic clamp, or surrogate methods such as the HOMA

index, the frequently-sampled intravenous glucose tolerance test or the insulin suppression test, appears in more than 76% of subjects, and is accompanied by compensatory hyperinsulinemia[112]. Although molecular mechanisms of IR are not yet entirely understood, abnormalities in insulin signalling have been explained[113]. Under normal conditions, insulin starts its action by binding to its specific cell surface receptor in peripheral tissues such as liver and skeletal muscle. The conformational changes of the insulin receptor induced by insulin binding to the extracellular alpha-subunit of the insulin receptor, causes the dimerization of neighboring receptors and the activation of the tyrosine kinase domain of the intracellular beta-subunit. Autophosphorylation of the beta-subunit itself, promoted by the onset of tyrosine kinase activity of insulin receptor, and the rapid phosphorylation of docking proteins, such as insulin receptor substrates -1, -2, -3 and -4, and some other proteins, comprising collagen homology proteins (shc) and SRC homology 2 (SH2), activates consecutively multiple intracellular signalling intermediates. In their phosphorylated forms, these proteins develop points of anchoring for intracellular proteins containing complementary SH2 domains, playing an important regulatory function in the insulin-signalling cascade. Specifically, the activation of Akt or protein kinase B, which plays an essential role in the mechanism of insulin action on GLUT-4 translocation, glucose transport, and the activation of NO synthase ("metabolic signalling pathway"), is determined by the interaction between insulin receptor substrate-1 proteins and phosphatidylinositol (PI) 3-kinase. On the contrary, the activation of Ras (predominantly through shc and, to a lesser degree, insulin receptor proteins), Raf, and mitogen-activated protein kinases (MAPK) ("growth signalling pathway") are implicated in the mitogenic, nonmetabolic, pro-inflammatory and proliferative effects of insulin[114]. A decreased activation of insulin signalling *via* the insulin receptor substrate-1/PI3-kinase (PI3K), can be showed in insulin-resistant animals and *in vitro* models. This reduction leads to a decreased glucose uptake, diminished NO synthesis, and reduced glucose utilisation in insulin target tissues pathway. Similar decrease in glucose transport is detected in the pancreatic beta cells, which induces a compensatory rise in insulin secretion. In spite of this, the MAPK-mediated insulin pathway persists unaffected. Under these conditions of hyperinsulinemia, this selective imbalance of the two signal transduction pathways can lead to a disproportionate proliferative/growth-promoting signal, while the normal transport of glucose and glucose homeostasis is conserved. Compensatory hyperinsulinemia stimulates in vascular smooth muscle and endothelial cells, an increased production of endothelin, PAI-1, proinflammatory cytokines and an augmented surface expression of adhesion molecules[115-118].

Homeostasis of blood vessels is conserved through the activation of endothelium-derived NO, stimulated by insulin. By rapid posttranslational mechanisms, which are mediated through PI3K/Akt signaling pathway, insulin augments the endothelial NO production by activating endothelial NO synthase Ⅲ (endothelial NOS)[119]. In IR states, the selective inhibition of the PI3K/Akt pathway detected in skeletal muscle from obese people and subjects with T2DM[120], and in the vasculature and the myocardium of obese Zucker rats, leads to endothelial dysfunction, with a consequent rise in the interaction between endothelial cells and leukocytes, an increase in vascular tone and BP, and a prothrombotic state. In this selective state, largely due to the ability of insulin to increase NO production, its physiological anti-atherogenic effects become proatherogenic[121].

Postprandial hyperglycaemia and glucose variability

Postprandial hyperglycaemia has been appeared to be related with an augmented risk of cardiovascular events in patients with and without T2DM[122-125]. Postprandial glucose excursions, especially when accompanied by increased postprandial TGs levels, are pathophysiologically related to augmented OE, systemic inflammation and endothelial dysfunction, all of which are associated to increases in atherosclerosis and cardiovascular events[126,127]. Postmeal hyperglycaemia is also linked to retinopathy, cognitive dysfunction in old people and specific cancers[128]. Relevantly, even in the setting of controlled fasting glucose levels, postprandial spikes in glucose powerfully improve both atherogenesis and cardiovascular events[122-125,129].

Two studies have examined the predictive strength of postprandial glycemia on cardiovascular events. The Intervention Diabetes Study[130], a prospective population-based multicentre trial, conducted in 1139 subjects, aged 30-55 years, newly diagnosed of T2DM, followed up for 11 years; showed that postprandial blood glucose was an independent predictor for death. However, this study did not consider HbA1c. On the other hand, the San Luigi Gonzaga Diabetes Study[122], conducted in 505 T2DM patients followed up for 14 years, indicated that both postprandial blood glucose and HbA1c predict cardiovascular events and all-cause mortality, showing the independent predictive power of postprandial glycemia on cardiovascular events after correction for HbA1c.

It has been shown that intensive control of hyperglycemia prevents macrovascular events and all-cause mortality in individuals with T2DM. A meta-analysis of 5 randomized controlled trials showed that, in T2DM subjects, intensive glycaemic control considerably decreases coronary events without an increased risk of death [131]. However, the specific effect of postprandial blood glucose control on cardiovascular events and mortality, is less clear. The following evidence is available: (1) Intervention with acarbose, a drug that diminish postprandial blood glucose excursions by delaying carbohydrate digestion in the small intestine, can prevent myocardial infarction and CVD in T2DM patients[132]. Moreover, in patients with impaired glucose tolerance, in Study to Prevent Non Insulin Dependent Diabetes Mel-

litus, acarbose was associated with a 49% relative risk reduction in the development of cardiovascular events[133]; (2) Nateglinide, a drug that lowers postprandial blood glucose by stimulating insulin secretion from the pancreas, was incapable in the Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research trial[134] to diminish cardiovascular events among persons with impaired glucose tolerance and established CVD; however, patients on Nateglinide presented an increase of 2-h postchallenge blood glucose[134]; and (3) The Hyperglycemia and its Effect after Acute myocaRdial infarcTion on cardiovascular outcomes in patients with T2DM trial, planned to compare the effects of prandial *vs* fasting glycemic control on risk for cardiovascular outcomes in subjects with T2DM after acute myocardial infarction, revealed that treating diabetic survivors of acute myocardial infarction with two distinct insulin regimens (prandial *vs* basal) achieved differences in fasting blood glucose, less-than-expected differences in postprandial blood glucose, and no difference in risk for future cardiovascular event rates[135].

Therefore, the role of postprandial glycemia as a predictor of cardiovascular events, and its importance as a treatment target, are issues to discuss.

These assessments had led to the concept of glucose variability. Recently, it has been suggested that blood glucose variability may contribute, even more than HbA1c, to the development of diabetes complications. However, the lack of consensus on the best approach to define the glucose variability, and difficulty of measuring it, are still unsolved problems. The relationship between glucose variability and OE, is an important physiopathological element for the development of the cardiovascular complications of diabetes. Glucose variability, thus, looks set to become the main target for future treatments for diabetes, aimed to reaching better efficacy in the metabolic control of diabetes and the prevention of complications related to it[136].

Microalbuminuria

The term microalbuminuria (MA), a urinary albumin excretion between 30 and 300 mg/24 h, has been introduced to identify subjects at increased risk of early cardiovascular death and progressive renal disease. In individuals with T2DM, MA is a prematurely clinical sign suggestive of vascular damage to the glomerulus. MA has also been currently reported as an important risk factor for CVD and remains the main and most widely used marker of diabetic renal damage in clinical practice. It is also a marker of organ dysfunction, and has been appeared to be associated with an increased risk of cardiovascular morbidity and mortality in T2DM patients[137]. At present, an increased albumin excretion is considered to be a renal symptom of generalized endothelial dysfunction[138]. According to different studies, the prevalence of MA is up to 19% in T2DM[139-142].

The epidemiology of MA shows a close association with systemic and glomerular endothelial dysfunction and with vascular disease. Damage to glycocalyx, a protein rich surface layer on the glomerular endothelium, probably represents the initial step in the development of diabetic MA[143].

MA is a marker for diabetic nephropathy. It also signifies CVD as well as nephropathy in T2DM. MA may precede T2DM, and forms one of the components of the IR/metabolic syndrome which confer a particularly high risk of cardiovascular deaths. Therefore, MA accounts for the increased risk of vascular disease in subjects with metabolic syndrome[144]. Other indicators of cardiovascular risk, such as markers of inflammation, are related with MA in population of patients with and without diabetes[145]. The existence of MA in people with T2DM is the most important early sign that we alert us to the onset of a systemic vascular disease, and associated target organ damage to the heart, the brain and the kidney. Their presence serves to recognize patients at risk of early cardiovascular death and advancement of kidney disease[146].

Patients with MA are at very high vascular risk and should share identical objectives of a vascular risk factor control as patients with overt CVD[147]. MA in patients with T2DM positively correlates with the severity of coronary atherosclerosis[148]. Reinhard *et al*[149] showed that half of asymptomatic patients with T2DM and MA, which received an intensive multifactorial treatment for cardiovascular risk diminution, had significant atherosclerosis in at least one vascular territory. They observed a higher prevalence of coronary atherosclerosis than carotid disease[149]. On the other hand, MA was higher in T2DM patients with silent myocardial ischemia[150].

The presence of MA also indicates that a low-level inflammatory process is ongoing. In hypertensive individuals, with or without diabetes, increasing MA is related with augmented levels of inflammatory markers, endothelial dysfunction and platelet activation[151]. Elevated plasma osteoprotegerin, a cytokine receptor, is an independent predictor of the presence of CVD in asymptomatic T2DM patients with MA[152], and CRP, a marker of inflammation, was an independent risk factor for development of nephropathy in T2DM patients[153]. Finally, D-dimer, a fibrin degradation product, is associated with MA in T2DM patients; this suggests that glomerular dysfunction is in part mediated by hypercoagulability[154].

Duration of diabetes[139], diabetes severity[139], uncontrolled hypertension[139,141,153,155-157], baseline levels of urinary albumin excretion > 12 mg/24 h[153], BMI[139,157], central obesity[139,140,155], high HbA1c[139,141,157], smoking habits[140,155,157], age of the patients[156], creatinine[141], CRP > 3 mg/L[153], as well as TGs and HDL-C[136,156] were independent risk factors for the development of MA in T2DM patients. These risk factors were independently associated with established MA. Population of normotensive subjects with T2DM and MA, female sex, was related with elevated risk of fatal and nonfatal CVD, independent of the traditional cardiovascular risk factors, the severity of nephropathy or existence of retinopathy, or health

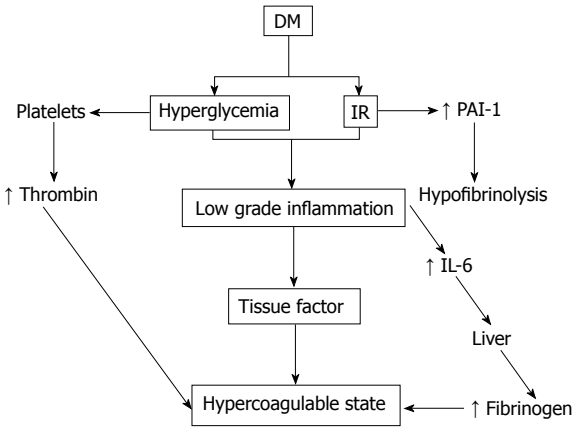

Figure 2 Prothrombotic mechanisms in diabetes mellitus. The hypercoagulable state related with diabetes mellitus is consequent to improved thrombin generation by platelets, impaired fibrinolysis due to increased levels of plasminogen activator inhibitor type 1 (PAI-1), and low-grade inflammation, that rise circulating levels of interleukin-6 (IL-6), fibrinogen, and tissue factor expression in vascular cells. DM: Diabetes mellitus; IR: Insulin resistance.

care utilization[158]; and a decreased estimated glomerular filtration rate and the occurrence of MA were each related with a near doubling of the prevalence of CVD, independently of classical cardiovascular risk factors and glycaemia control in subjects with T2DM[159].

Carotid stiffness, quantified by quantitative carotid stiffness, a local functional measurement of the arterial wall, is augmented in T2DM patients with MA[160]. MA is also independently linked with arterial stiffness and vascular inflammation in individuals with newly diagnosed T2DM[161], but not with carotid intima-media thickness[161,162], with emphasizes the significance of proactive clinical investigations for atherosclerotic complications in subjects with MA in newly diagnosed DM. On the other hand, patients with MA have more severe angiographically detected CAD than those without MA[163]. Thus relative is independent of other risk factors and is particularly evident in patients with T2DM[164].

In conclusion, MA is a marker for diabetic nephropathy. It also signifies CVD in T2DM. MA is predictive, independent of classical risk factors and all causes of mortality in T2DM individuals. Determination of MA has been shown to be helpful to recognize patients with T2DM at high risk of renal and CVD. MA is correlated with higher cardiovascular mortality, especially in diabetics, but the direct relationship between MA and vascular wall properties is still not clear.

Haematological and thrombogenic factors

Atherothrombosis, defined as the formation of a thrombus on a pre-existing atherosclerotic plaque, is the leading cause of mortality in the Western world. Diabetes has been recognised as an independent risk factor and atherothrombosis accounts for the 80% of deaths in these patients[165,166]. It is the result of the progression of atherosclerosis, and its major manifestations are sudden cardiac death, myocardial infarction, stroke and peripheral arterial ischemia[167].

Diabetes is related with a hypercoagulable state, which is more pronounced during the postprandial period. Hyperactivated platelets at injured endothelial interfaces act, together with an improved availability of thrombotic precursors, decreased coagulation inhibitors and diminished fibrinolysis[168]. The UKPDS clearly showed that macrovascular events in patients with T2DM accounted for more than 50% of total mortality[169]. Atherosclerosis develops more quickly and aggressively in diabetes, and leads more frequently to thrombotic events due to the interaction between the vascular wall and hypercoagulability[170,171].

In DM, the activation of the intrinsic coagulation pathway occurs more easily and fibrinolysis diminishes[172]. The increased platelet activity signifies increased adhesion and aggregation in diabetic patients (Figure 2). Individuals with various stages of diabetes were showed to have increased numbers of CD62P-positive and CD63-positive platelets (activated platelets) compared to healthy subjects. This increase in circulating activated platelets is not associated with glycaemic control improvement thereby intensifying insulin therapy. Surprisingly this increase in CD62P-positive platelets can also be found in healthy, first-degree relatives of patients with T1DM. Additionally, significant increments in basal thromboxane B[164] are seen in the platelets of both type T1 and T2DM, both in patients with an absence of vascular complications, as well as those with good diabetic control.

Flow cytometry has revealed that a large, hyperactive platelet subpopulation circulates in patients with DM, at a similar level to patients who have experienced a myocardial infarction. This suggests that the increased aggregation potential of these platelets lowers their activation threshold, thus contributing to the augmented incidence of acute cardiovascular events in DM[173].

Apart from platelet hyperactivity, DM also predisposes the coagulation system to other disorders[174]. Fibrinolysis is a natural defence system against thrombosis. Under physiological conditions, there is a balance between plasminogen activators and inhibitors; however, an imbalance can be caused by a reduction in the tissue plasminogen activator levels or an increase in the PAI-1 levels. This prethrombotic state in diabetic patients has been explained by multiple hypotheses. One such hypothesis is based on various studies showing the high levels of PAI-1 found in diabetic patients[175,176]. High concentrations of PAI-1 have been implicated with an increase in cardiovascular morbidity and mortality with age. A search was made for the relation of PAI-1 with various factors such as age, gender and ethnicity in subjects with T2DM and stable CAD enrolled in the BARI 2D study. The results of this study concluded that in subjects with T2DM and stable CAD, the levels of PAI-1 antigen and its activity were paradoxically lesser with advancing age; and in contrast, D-dimer ($P < 0.0001$) was increased, revealing elevated fibrinolysis. These results may indicate a protective phenomenon resulting in an improved survival in some older people with DM that endowed them with longevity enough to permit them to participate in the

BARI 2D study[177].

Another hypothesis in this prethrombotic state is that hyperglycaemia permits the protein glycosylation process, such as the fibrinogen, which affects the clot's physiological structure, and thus it is more resistant to plasmin degradation.

DM is also associated with increased plasma fibrinogen, which is considered as another cardiovascular risk factor[12,178]. This increase in fibrinogen is also associated with other vascular risk factors such as old age, increased BMI, smoking, total cholesterol and TGs. Fibrinogen has been extensively studied by many researchers, and a connection between the amount of fibrinogen and fibrin present in the vascular wall, the fibrinogen plasma concentration and the severity of atherosclerosis has been established. This association has been shown to be more evident in patients with diabetes[179,180]. Furthermore, an elevated concentration of fibrinogen has been found in diabetic patients with albuminuria. Some authors believe that the increased levels of fibrinogen, factor Ⅶ and von Willebrand factor which have been found in DM patients serve as predictors of coronary atherosclerosis and cardiovascular risk factors[181]. This association supports the fact that diabetic patients develop cardiovascular complications more frequently than the healthy population.

Inflammation: C-reactive protein

Atherosclerotic CHD and other forms of CVD are the main cause of mortality in T2DM, as well a major contributor to morbidity and lifetime costs. When diabetes occurs in subjects with established CAD, absolute risk for future events is very high. Inflammation has been involved in the pathogenesis of CVD, T2DM, and cancer. Different biochemical parameters may be utilised for the evaluation of CVD risk in T2DM patients of different age[182]. CRP is an acute-phase protein produces in the liver; its release is stimulated by cytokines (interleukin 6 and tumour necrosis factor alpha). Increased levels of it are related with the presence and severity of CAD and renal impairment in individuals with T2DM[183]. Although the determination of high-sensitivity CRP (hs-CRP) level represents an interest in the screening of CVD in T2DM patients[184].

Increased concentrations of hs-CRP are associated with IR, T2DM and the development of CVD. In particular, inflammation strongly linked with endothelial dysfunction is accepted as one of the cardiovascular risk factors clustering in the IR syndrome or metabolic syndrome. Moreover, low-grade inflammation might play an important role in the pathobiology of the metabolic syndrome[185,186]. The exact mechanism linking IR and inflammation remain unclear. Several studies have drawn attention to the finding of increased levels of hs-CRP in T2DM patients with features of the metabolic syndrome[187-190]. The elevation of hs-CRP was strongly correlated with BMI, serum lipids, fasting glucose and WC[191-197], features of the metabolic syndrome, indicating potential roles of obesity and abdominal obesity in the development of inflammation associated with the metabolic syndrome in T2DM patients. The strong association between IR and inflammation in atherogenesis insinuates that therapies that address both parameters, such thiazolidinediones may have benefits in decreasing diabetes-related macrovascular complications[198].

Serum concentration of hs-CRP is a good biomarker of chronic low-grade inflammation and is an established prognostic marker in acute coronary syndrome. In subjects with DM, the presence of high plasma levels of hs-CRP are predictive for fatal and non-fatal CHD[199,200]. Although suffering from an acute CAD, patients with T2DM have a poor outcome compared with non-diabetic patients, in part explained by a persistent endothelium-dependent dysfunction and inflammatory activity in these patients after acute myocardial infarction[201]. Finally, hs-CRP was related with silent myocardial ischemia, and might help to detect silent myocardial ischemia in diabetic patients[202].

On the other hand, in T2DM patients hs-CRP was an independent risk factor for CHD deaths[203]. In a case control study including 60 T2DM subjects with normal lipid profile and 60 age and sex-matched healthy controls, hs-CRP was an independent cardiac risk predictor even with normal lipid profile and can help measure additional risk[204]. Moreover the Diabetes Heart Study (DHS) documents the utility of hs-CRP in predicting risk for all-cause mortality in 846 European Americans with T2DM, and supports its use as a screening tool in risk prediction models[205]. However, in acute coronary syndrome few studies found no significant differences in hs-CRP between patients with and without diabetes[206]. Khatana *et al*[207] have found that hs-CRP may not be suitable to predict changes in cardiovascular risk among diabetic patients, and should not be a surrogate for achieving evidence based goals in traditional cardiovascular risk factors; in the Prospective Evaluation of Diabetic Ischaemia Heart Disease by Coronary Tomography Study, there was a negative association between coronary artery calcification score, obtained by electron beam tomography, and CRP in T2DM patients[208]; and the Irbesartan Diabetic Nephropathy Trial with baseline data obtained from 722 diabetic nephropathy patients showed a lack of association between hs-CRP and specific established or emerging cardiovascular risk factors[209].

Diabetic patients with MA and hypertension had more frequent association with increased marker of inflammation such hs-CRP[210]. The correlation found between hs-CRP levels and albuminuria in T2DM patients[211] suggest that the inflammatory process plays a role in diabetic nephropathy patients. However, in these patients CRP does not add predictive information above and beyond that offered by traditional established risk factors[212].

Several large prospective studies have proved that baseline levels of hs-CRP are an independent predictor of cardiovascular events among apparently healthy individuals. However, prospective data on whether hs-CRP predicts cardiovascular events in diabetic patients

are limited so far. The Prevention of Renal and Vascular End stage Disease study, a prospective population-based cohort study in the Nederland's, including 8592 participants[213] show that elevated hs-CRP, has added value to the present metabolic syndrome defining variables in predicting new onset CVD. In a prospective cohort study, baseline hs-CRP level is associated with increased first cardio-cerebral vascular event in the population with DM[214]. In the Casale Monferrato Study[215], hs-CRP measurement is independently related with short-term mortality risk in T2DM patients, even in normoalbuminuric individuals, and in those without a prior diagnosis of CVD; and in the Chennai Urban Rural Epidemiology Study (CURES)[216], an ongoing population-based study conducted on 150 subjects selected from the CURES, hs-CRP demonstrated a solid association with CAD and diabetes even after adjusting for age and gender. Finally, in a prospective study a cohort of 746 American men aged 46-81 years who were free of CVD at the time of blood collection in 1993-1994 were followed[217]. In this study elevated plasma levels of hs-CRP were related with an improved risk of incident cardiovascular events among diabetic men, independent of currently established lifestyle risk factors, blood lipids and glycaemic control.

On the other hand, in the recent ADVANCE study[218], the authors deduce that interleukin-6 levels but not CRP or fibrinogen levels, add significantly to the prediction of macrovascular events and mortality in patients with T2DM who have baseline CVD or risk factors.

In conclusion, the serum levels of hs-CRP, which is a marker of systemic inflammation and a mediator of atherosclerotic disease, have been correlated with the risk of CVD in T2DM patients. The determination of it is very important as screening of CVD in T2DM patients.

Homocysteine and vitamins

Homocysteine (HC) is a sulphur-containing essential amino acid derived from methionine. Vitamins B6, B12 and folic acid act as coenzymes in the metabolism of methionine and HC, and individual deficiency may cause hyperhomocysteinemia (HHC)[219]. Therefore, a negative correlation exists between HC plasma levels and vitamins B6, B12 and folic acid levels[220]. The HC plasma levels are higher in men, in women they increase after the menopause, and in both sexes they rise with aging[219]. An increase in HC levels has also been described in chronic kidney disease through a mechanism that is still not entirely understood, although it has been related to decreased renal clearance and metabolism and/or a descent in the extrarenal metabolism resulting from retained inhibitory substances[221].

In T2DM subjects, elevated HC levels have been related with a rise in the risk of suffering from cardiovascular events, independent of other risk factors[222,223], such as age and renal function[223]. The close relation between HC and CVD confirms the atherosclerotic role in the same[224]. For some authors, higher HC levels are consistent not only with aging and the male gender, but also in line with the development of DM[225]. HC levels do not appear to be related with anthropometric indices such as weight, BMI, percentage of fat mass and triceps skin fold[226].

On the other hand, the HC could play an etiologic role in the pathogenesis of T2DM, promoting OE, systemic inflammation and endothelial dysfunction[227]. HC seems to be the cause of increased mortality in T2DM subjects[223], and some authors consider it as a predictor of mortality[228]. The highest HC levels have been found in diabetic patients who have suffered several cardiovascular events[222].

The role of HC as a cardiovascular risk factor in DM is unclear. The poor metabolic control of the T2DM patients appears to have a predominant role. There exists a positive correlation between the HC levels and those of HbA1c, and a negative correlation with those of insulin[229]. On the other hand, a decline in HC levels has been observed in diabetic patients with a high cardiovascular risk and an elevated intake of foods high in folate, and vitamins B6 and B12[230]. Furthermore, an important predictor of cardiovascular risk in T2DM is arterial compliance which may not only be associated with age, but also with HC levels and renal function parameters[231].

The HHC as a cardiovascular risk factor includes CHD, both in the general population and in the diabetic population, although the role it plays on T2DM is unknown. However, the HHC in plasma is closely related to the development of CAD[232]. Thus, elevated HC levels have been found in patients with CHD, closely correlated with the occurrence of the same in the presence of decreased levels of folic acid and HDL-C[233].

Silent myocardial ischemia is one of the most frequent causes of mortality in the United States and it not only affects the diabetic population. Traditional risk factors have been identified such as T2DM itself, hypertension, dyslipidaemia and smoking, but there are also a series of novel factors such as lipoprotein (a), CRP and HC that can help improve the evaluation of patients with this disease[233]. In patients with T2DM, silent myocardial infarctions have been associated with these novel cardiovascular risk factors such as increased HC[234]. The HHC is related with increased mortality in T2DM patients suffering from CAD, without, however, being a predictor factor of cardiovascular mortality[235].

MA is a predictor of CVD and shows a close relationship with HC. The reason for this association is unknown; however it could be in the origin of MA. There are studies that show a relationship between HC and MA, irrespective of T2DM and hypertension[236]. In T2DM subjects with a high prevalence of peripheral arterial disease and nephropathy, there exists a relationship between the levels of HC and those of MA[226]. Finally, HHC is considered as a risk factor for the development of peripheral arterial disease in T2DM individuals over 65 years of age[237].

HHC is linked with the risk of developing peripheral

and autonomic neuropathy. In T2DM, HC is associated with neuropathy developing through an ischemia. The rise of HC appears to be independently associated with autonomic neuropathy, showing no association with peripheral diabetic neuropathy. For each increase in HC, there is a 7.1% increased risk of developing autonomic neuropathy[238].

In a study of T2DM subjects *vs* non diabetic control subjects, HC levels were found as elevated as those found with preproliferative retinopathy and glaucoma, suggesting that HC was a risk factor for the development of microvascular lesions in these subjects[239]. Small HC elevations in patients with diabetic retinopathy have been associated with capillary and endothelial dysregulation, in which the HHC could be an important risk factor for the development of a macular oedema[240].

An increase in HC levels has been described together with a decrease in levels of folic acid and vitamin B12[241]. However, taking folic acid, and vitamins B12 and B6 supplements with the aim of reducing HC levels does not decrease the risk of developing CVD[237]. Vitamin B12 deficiency together with an elevation of HC will predispose towards an augmented risk of cardiovascular morbidity and mortality in T2DM subjects. Vitamin B12 supplementation in these patients will not reduce the cardiovascular risk[242].

As regards vitamin A, it is capable of affecting the inflammatory mechanisms and the immune function and therefore be associated to CVD. However, there does not appear to be a relation to the cardiovascular risk, as variations of the same are not found in T2DM[243,244]. Neither has an association been found between the zinc levels and cardiovascular risk with the HbA1c levels in T2DM patients[244].

The actions of vitamin D are mediated by binding to a specific nuclear vitamin D receptor (VDR)[247]. Allelic variations of the *VDR* gene are related with improved risk of CAD in T2DM patients[245]. The hypothesis that vitamin D might protect against vascular disease, comprising atherosclerosis and endothelial dysfunction, is postulated since it has been observed that the VDR is also expressed in the vasculature[246]. An increased production of NO, the inhibition of macrophage to foam cell formation, or a decreased expression of adhesion molecules in endothelial cells, might mediate the vascular protective actions of vitamin D[247-249]. Both endothelial dysfunction and increased arterial stiffness[246,250], and more recently cardiovascular risk factors including T2DM[251], and an elevated risk of CVD[252] are related with low vitamin D levels. Of published observational studies, most have shown that lower levels of vitamin D are related with a high incidence of cardiovascular events and mortality[253-257]. Even asymptomatic CAD was associated with lower vitamin D levels in high risk T2DM patients, as observed in a recent observational study[258]. On the other hand, in T2DM patients, severe vitamin D deficiency predicts improved risk of all-cause and cardiovascular mortality, independent of urinary albumin excretion rate and conventional cardiovascular risk factors[259], and vitamin D deficiency appears to be a significant risk factor for T2DM severity and associated cardio-metabolic risk[260]. Furthermore, in a double-blind, parallel group, placebo-controlled randomized trial, a single large dose of 100000 IU vitamin D2 improves endothelial function in patients with T2DM and vitamin D insufficiency[261]; and in a prospective study, vitamin D supplementation (2000 IU/d) in patients with T2DM on different therapeutic regimens, those patients on insulin in combination with other drugs was the group that benefited the most as compared with other groups in terms of improving cardiovascular risk[262]. Thus, we can conclude that in T2DM vitamin D deficiency is an independent cardiovascular risk factor, but whether vitamin D supplementation can significantly improve cardiovascular outcomes is yet largely unknown. However, early intervention may be considered to improve prevention of T2DM related cardiovascular complications.

It has been believed for years that caffeine, one of the substances most used worldwide and included in coffee, tea, energy drinks and chocolate, increases coronary risk, hypertension and HC concentrations. However their high consumption could modulate insulin sensitivity and blood glucose levels, and in the long term it may reduce the incidence of T2DM[263]. Therefore caffeine would not have any adverse cardiovascular effects, as it demonstrates an antioxidant capacity, and presents an inverse risk association with regard to T2DM[264].

Chronic alcoholism may produce an HC plasma increase due to nutritional deficiencies associated with the said habit[265,266]. An association between alcohol and the development of atherosclerosis has been observed in patients with T2DM. Alcohol consumption and HHC together could explain the occurrence of atherosclerosis in diabetic subjects[267].

Finally, regarding treatments for T2DM, metformin appears to reduce folic acid levels in the blood, which in the long-term would raise HC levels. Folate management in these patients would reduce the levels of the same[268].

Erectile dysfunction

Men with DM have a higher prevalence of erectile dysfunction (ED) compared with the general population[269]. In these individuals, the prevalence of ED augments with age and duration and severity of disease[269,270]. ED and atherosclerosis are frequent complications of DM[271]. There are close relations between ED and atherosclerosis in patients with T2DM, and ED might serve as a clinical marker for coronary, peripheral, or cerebrovascular diseases in these subjects[272]. Several studies have found a positive correlation between ED and the risk of cardiovascular events[273,274]. The total cardiovascular risk increases severity of ED in T2DM patients without having overt CVD[275]. A cohort study concluded that the presence of ED in men with T2DM and without clinically overt CVD predicted CHD[276], and another study indicates that ED appears to be robustly and independently

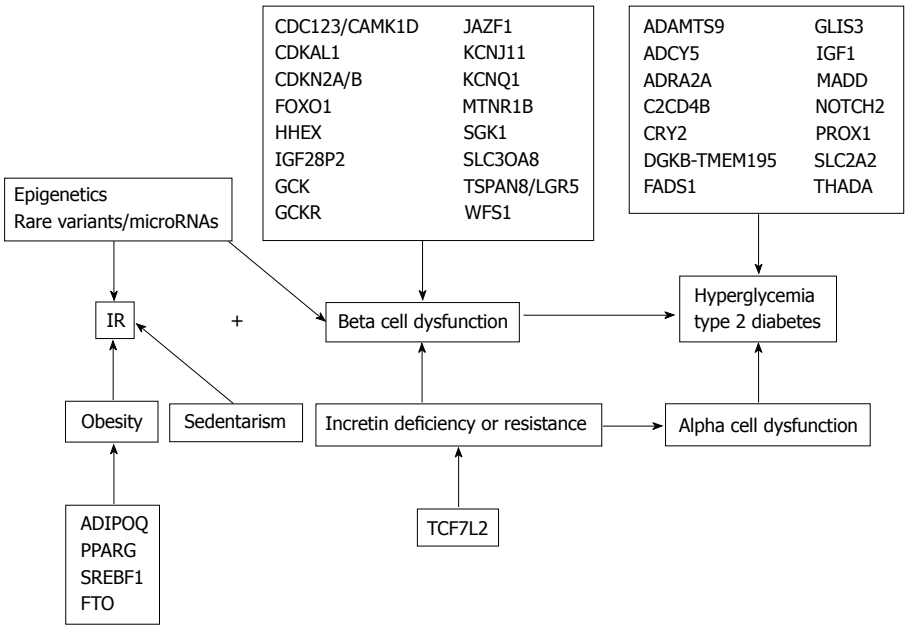

Figure 3 Possible mechanisms of confirmed and potential risk single-nucleotide poly morphisms in type 2 diabetes. Many single-nucleotide poly morphisms (SNPs) affect pancreatic beta-cell function. Gene symbols represent SNPs in or near these gene loci. Likely epigenetic alterations microRNAs and/or rare genetic variants also have a critical role. The mechanisms by which some genes increase the risk of diabetes are not yet know. CDC123: Cell division cycle 123; CAMK1D: Calmodulin-dependent protein kinase 1D; CDKAL1: CDK5 regulatory subunit-associated protein 1-like 1; CDKN2A/B: Distal to the genes cyclin-dependent kinase inhibitors 2A; FOXO1: Fork head box protein O1; HHEX: IDE-near hematopoietically expressed home box and insulin degrading enzyme; IGF2BP2: IGF2 mRNA binding protein 2; GCK: Glucokinase; GCKR: Glucokinase regulator; JAZF1: Juxtaposed with another zinc kinger protein 1; KCNJ11: Potassium inwardly-rectifying channel, subfamily J, member 11; KCNQ1: Potassium voltage-gated channel, KQT-like subfamily, member 1; MTNR1B: Melatonin receptor 1B; SGK1: Serum/glucocorticoid regulated kinase 1; SLC30A8: Solute carrier family 30 (zinc transporter) member A8; TSPAN8: Tetraspanin 8; LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; WFS1: Wolfram syndrome 1; ADAMTS9: Disintegrin and metalloproteinase with thrombospondin motifs 9; ADCY5: Adenylate cyclase 5; ADRA2A: Adrenergic, alpha-2A-, receptor; C2CD4B: C2 calcium-dependent domain containing 4B; CRY2: Cryptochrome 2; DGKB: Diacylglycerol kinase beta 90 kDa; TMEM195: Transmembrane protein 195; FADS1: Fatty acid desaturase 1; GLIS3: GLIS family zinc finger 3; IGF1: Insulin-like growth factor 1; MADD: MAP kinase-activating death domain; NOTCH2: Notch homolog protein 2; PROX1: Prospero-homebox 1; SLC2A2: Solute carrier family 2, member A2; THADA: Thyroid adenoma associated; ADIPO: Adiponectin; PPARG: Peroxisome proliferator-activated receptor-gamma; SREBF1: Sterol regulatory element-binding transcription factor 1; FTO: Fat mass and obesity-associated; TCF7L2: Transcription factor 7-like 2; IR: Insulin resistance.

related with silent CAD in apparently uncomplicated T2DM subjects[272]. Moreover, a meta-analysis of observational studies concludes that the presence of ED was related with an elevated risk of cardiovascular events in diabetic men[277].

Finally, a recent paper suggests that vitamin D deficiency is closely related with both ED and CVD, and the authors postulate that optimizing serum vitamin D levels through vitamin D supplementation helps delay the onset of ED[278].

Genetics and epigenetics

T2DM is an independent risk factor for developing CVD with the relative risk of CVD mortality of 4.9 in women and 2.1 in men, relative to non-diabetics subjects[165,279]. Genetic and environmental factors contribute to this risk. In the past decade, genome-wide association studies had elevated the number of common single-nucleotide polymorphisms, which confirmed the relationship between T2DM and CVD (Figure 3).

Haptoglobin polymorphisms and CVD in T2DM: Haptoglobin (HP) has been involved in both T2DM, and T2DM related CVD[280,281]. HP binds to ApoA1 in the same location as lecithin-cholesterol acyltransferase (LCAT); this lead to a decrease LCAT activity and consequently limiting HDL maturation. This inhibits reverse cholesterol transport causing HDL to become proatherogenic[282].

Several studies have investigated HP polymorphisms and CVD risk in T2DM. In 2002 Levy *et al*[283] reported an OR of CVD events in diabetes five times greater with the HP 2-2 phenotype, than with HP 1-1 in a study that involved 206 CVD patients and 206 CVD controls (146 and 93 were affected by T2DM, respectively, as part of the Strong Heart Study). In 2004, a subsequent study by Levy *et al*[284] involved 3273 subjects in the Framingham Heart Study, however only a subset of 433 patients were affected with T2DM, and of these, only 86 had a history of prevalent CVD. Finally, a 2003 study in patients with acute myocardial infarction reported that individuals with T2DM and the HP2 allele had improved mortality following acute myocardial infarction, compared to subjects with T2DM and the HP 1-1 genotype (included only 224 T2DM affected patients)[285]. The DHS is a study of the genetic and epidemiological causes of CVD in patients with T2DM. In a sub-study of 1208 subjects from the DHS, the HP 2-2 genotype was associated with

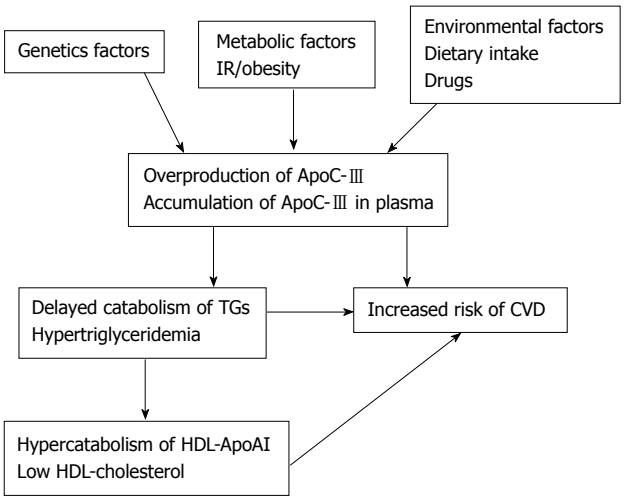

Figure 4 Dysregulation of apolipoprotein C-Ⅲ transport as a central cause of cardiovascular disease. Apo: Apolipoprotein; TGs: Triglycerides; HDL: High density lipoprotein; CVD: Cardiovascular disease; IR: Insulin resistance.

increased carotid intima-media thickness[286].

Apolipoprotein E gene polymorphism and risk of CVD in T2DM: ApoE plays an important role in lipid metabolism as a ligand for many cell-surface receptors comprising the LDL receptor, LDL-receptor related protein and VLDL receptor[287]. Human ApoE is genetically controlled by three alleles (e2, e3, and e4) at a single gene locus in chromosome 19; these code for three isoforms (E2, E3, and E4) and thus determine the six genotypes (e2/2, e4/2, e3/2, e3/3, e4/3, and e4/4)[287]. ApoE ε2 allele has been reported to be related with higher plasma levels of ApoE, reduced plasma levels of LDL-C and lower risk of CAD[288], while ApoE ε4 is related with lower plasma level of ApoE, elevated plasma levels of total cholesterol, LDL-C, VLDL-C, and greater risk of CAD when compared to ApoE3 homozygotes[289]. In diabetic population, Apoe4 allele is related with the risk of CAD[290,291], augmented occurrence of exercise-induced silent myocardial ischemia[292], impairment of endothelium-dependent artery dilation[293], and CAD death[294].

Genetic factors in the overproduction of Apolipoprotein C-Ⅲ and the risk of CVD in T2DM: Apolipoprotein C-Ⅲ (ApoC-Ⅲ) plays an important role in regulating the metabolism of TGs-rich lipoproteins (TRLs). ApoC-Ⅲ is an inhibitor of lipoprotein lipase and of TRLs remnant uptake by hepatic lipoprotein receptors. Elevated ApoC-Ⅲ, may cause accumulation of plasma TRLs lead to hypertriglyceridaemia (Figure 4).

The APOC3 gene exists in a gene cluster with the *ApoAIV* and *ApoA I* genes on chromosome 11q23[295]. ApoC-Ⅲ expression is down-regulated, in part, by insulin *via* the promoter insulin response element on the *APOC3* gene[296]. This indicates that ApoC-Ⅲ expression can be regulated by insulin sensitivity[297]. IR may blunt the sensitivity to the normal insulin-mediated suppression of *ApoC-Ⅲ* gene expression. The transcription of *APOC3* gene is also mediated by peroxisome proliferator activated receptors (PPAR)[298]. The induction of PPAR, principally the PPAR-α form, decreases *APOC3* gene expression[299,300].

Several studies reveal that naturally occurring sequence variation in APOC3 genes affects plasma ApoC-Ⅲ (and TGs) concentrations in humans. The APOC3 promoter variants at positions -455 and -482 have been studied more extensively because they relate to responsiveness to insulin. Moreover, there is increasing evidence showing the possibility of interactive effects between the *APOC3* gene variant and other environmental factors such as dietary intakes or smoking[301,302].

Epigenetics and the risk of CVD in T2DM: There is evidence linking epigenetic factors to various diseases such as DM and CVD[303]. Epigenetic factors could be an important mediator between DM, CVD and chronic inflammatory response, and, by different types of reactions such as acetylation and methylation, could mediate the interaction between genes and environment resulting in activation, repression or silencing the genetic transcription (Figure 5). In particular, DNA methylation has been linked to several cardiovascular-related biomarkers, including HC and CRP[304]. Hyperglycaemia may induce epigenetic changes of genes involved in vascular inflammation. Poor glycemic control increases nuclear transcription factor-κB (NF-κB), which regulates the expression of genes involved in inflammatory diseases, including atherosclerosis and diabetic complications[305], activity in monocytes and gene expression of inflammatory cytokines[306,307]. Moreover, in human aortic endothelial cells, the excess of reactive oxygen species resulting from a transient exposure to hyperglycaemia (16 h) can induce monomethylation of lysine from histone 3, increasing the expression of the subunit p65 of NF-κB[308], responsible for the increased transcription of VCAM-1, monocyte chemoattractant molecule 1, and some inflammatory proteins like interleukin-6, ICAM-1, and NOS, that are associated to hyperglycaemia-induced arterial pathology[307]. Epigenetic changes in the NF-κB p65 promoter induced by transient hyperglycaemia, which persist for 6 d during culture at normal glucose levels, can be regulate for two histones: a histone methylase and a histone demethylase[309]. In fact, the hyperglycaemic memory could be explained by epigenetic changes induced by transient hyperglycemia. Epidemiological studies insinuate that hyperglycemia may induce epigenetic changes of proinflammatory genes, which subsequently regulate gene expression and thereby the development of vascular inflammation[310].

PERSPECTIVES AND CONCLUSIONS

The complex interaction of risk factors in T2DM make it necessary to apply a holistic approach to the management of this chronic disorder, and a comprehensive care plan should therefore include modification of all cardio-

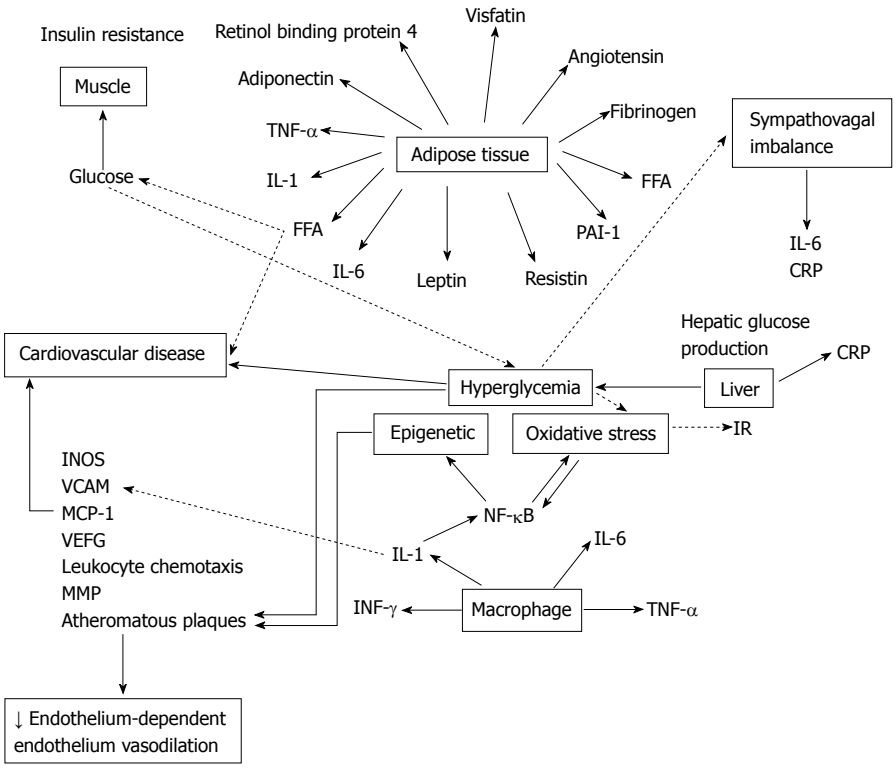

Figure 5 Pathogenesis of cardiovascular disease in diabetes. The mechanisms implicated in the pathogenesis of cardiovascular disease in diabetes comprehend epigenetic changes and intracellular metabolic changes that result in oxidative stress, low-grade inflammation, and endothelial dysfunction. CRP: C-reactive protein; FFA: Free fatty acids; INOS: Inducible nitric oxide synthase; IL-1: Interleukin 1; MCP-1: Monocyte chemoattractant molecule 1; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor kappa-β; PAI-1: Plasminogen activator inhibitor-1; VCAM: Vascular cell adhesion molecule; VEFG: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α; INF-γ: Interferon-γ; IR: Insulin resistance.

vascular risk factors. Targeting multiple markers of CVD risk hopefully offers the best chance of improving CVD outcomes.

There are consistent evidences that optimal glycaemic control, along with control of hypertension, dyslipidaemia, smoking cessation, and weight loss are necessary for reducing cardiovascular risk in T2DM patients. Cardiovascular benefits are obtained if the control of traditional cardiovascular risk factors begins early in subjects with short duration of DM and low cardiovascular risk. On the contrary, in elderly subjects with long duration of DM, exposed to hyperglycemia for a long time, and high cardiovascular risk, the same is not true. This beneficial or harmful effect could be explained by the hypothesis called as metabolic memory, in which the effect of the early glycemic exposure environment is imprinted in target organs, resulting in long-term protective or deleterious long-term effects.

In recent years there have been major advances of the influence of non-traditional risk factors for CVD in DM. This knowledge should gradually lead to the development of more effective therapeutic strategies to prevent cardiovascular events. Currently there is no evidence that routine monitoring of these risk factors in diabetic patients leads to better diagnostic and therapeutic results. Nor is there solid evidence to justify screening for subclinical atherosclerosis in asymptomatic subjects with DM.

Further work is needed to understand the impact of epigenetic changes of complications of T2DM, which can lead to the development of new therapeutic strategies for these patients. Research should focus on the factors that lead to dysfunction and failure of islet, particularly those acquired at an early age because they can be prevented. Epigenetic regulation of metabolic genes may be one of the fields of research.

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P- Reviewer: Collino M, Okumura K, Ray S, Yin JY
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Clinical Diabetes

2015 First Edition
bpgoffice@wjgnet.com

ISBN 978-0-9861420-0-0

Possible contribution of (pro)renin receptor to development of gestational diabetes mellitus

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Received: July 24, 2014 Revised: August 21, 2014
Accepted: October 23, 2014
Published online: December 15, 2014

Abstract

(Pro)renin receptor [(P)RR], a receptor for renin and prorenin, was first cloned in 2002. Since then, the pathophysiological roles of (P)RR have been growing concerns. (P)RR binds renin and prorenin, with two important consequences, nonproteolytic activation of prorenin, leading to the tissue renin-angiotensin system activation and the intracellular signalings. It is now also known to play an important role as vacuolar H^+-ATPase associated protein, involving in Wnt signaling, main component of embryonic development. Extracellular domain of full-length (P)RR is cleaved in golgi-complex forming soluble (P)RR [s(P)RR]. The s(P)RR is now possible to be measured in human blood and urine. It is now measured in different pathophysiological states, and recent study showed that elevated plasma s(P)RR levels in the early stage of pregnancies are associated with higher incidence of gestational diabetes mellitus later in the pregnancies. Plasma s(P)RR levels of neonates are known to be higher than that of adults. It was also shown that, increased s(P)RR concentrations in cord blood, associated with a lower small for gestational age birth likelihood. These data suggests the involvement of (P)RR in embryo's growth. In this review article, we attempt to figure out the possible pathophysiological roles of the (P)RR in maternal glucose intolerance and embryo's growth, through reviewing previous studies.

Key words: (Pro)renin receptor; Gestational diabetes mellitus; Embryonic growth; Renin-angiotensin system; Vacuolar H^+-ATPase; Wnt signaling

Core tip: Prorenin receptor [(P)RR] binds (pro)renin, and leads to the activation of tissue renin-angiotensin system and intracellular signalings. It also plays an important role as vacuolar H^+-ATPase associated protein, involving in Wnt signaling. Elevated plasma soluble (P)RR [s(P)RR] levels in the early stage of pregnancies are associated with higher incidence of gestational diabetes mellitus (GDM) during the third trimester. Also, elevated s(P)RR levels in cord blood, associated with a lower small for gestational age birth likelihood, suggesting the involvement of (P)RR in embryo's growth. Here we attempt to elucidate the possible pathophysiological roles of the (P)RR in GDM.

Original sources: Bokuda K, Ichihara A. Possible contribution of (pro)renin receptor to development of gestational diabetes mellitus. *World J Diabetes* 2014; 5(6): 912-916 Available from: URL: http://www.wjgnet.com/1948-9358/full/v5/i6/912.htm DOI: http://dx.doi.org/10.4239/wjd.v5.i6.912

INTRODUCTION

(Pro)renin receptor [(P)RR], a receptor for (pro)renin, was first identified in 2002[1]. The C-terminal domain of this receptor had been previously described as ATP6AP2 protein, which associated with a vacuolar H^+-ATPase (V-ATPase)[2], a proton pump essential for acidification of

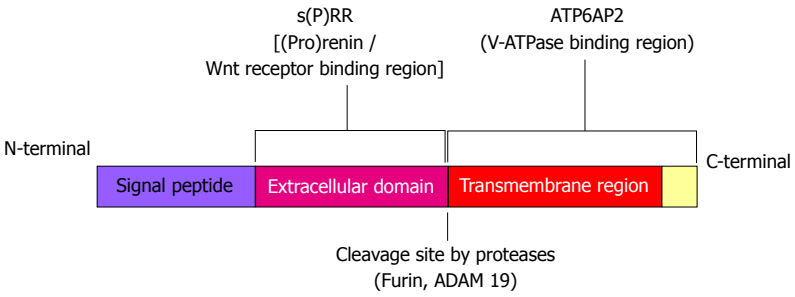

Figure 1 Structure of (pro)renin receptor. s(P)RR: Soluble (pro)renin receptor; V-ATPase: Vacuolar H^+-ATPase; ADAM 19: A disintegrin and metalloproteinase 19.

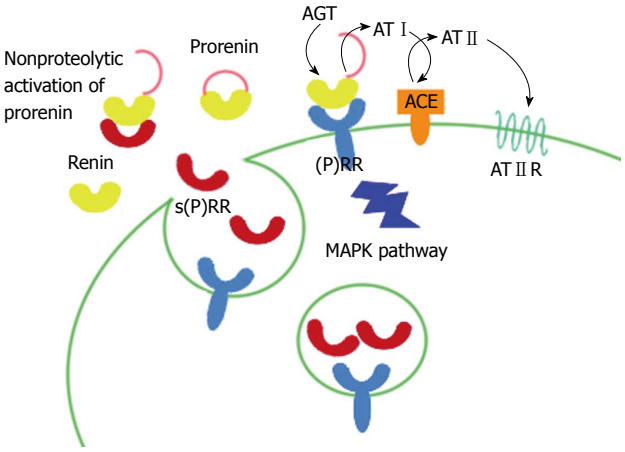

Figure 2 (Pro)renin receptor and (pro)renin. (P)RR: (Pro)renin receptor; s(P)RR: Soluble (P)RR; AGT: Angiotensinogen; AT Ⅰ: Angiotensin Ⅰ; AT Ⅱ: Angiotensin Ⅱ; ACE: Angiotensin converting enzyme; AT Ⅱ R: AT Ⅱ receptor; MAPK: Mitogen-activated protein kinase.

intracellular compartments. (P)RR consists of 350-amino acid with a single transmembrane domain and is known to exist in different molecular forms. Some exist as a full-length integral transmembrane protein, some as soluble (P)RR [s(P)RR] composed of extracellular domain, and other as truncated form composed of the transmembrane and cytoplasmic domains[3] (Figure 1).

When prorenin binds to (P)RR, a conformational change occurs in the prorenin molecule and gains full enzymatic activity without passing through proteolytic cleavage to renin[4]. Of different molecular forms of (P)RR, full-length and s(P)RR have a capacity of binding renin and prorenin. Thus, prorenin which is bound to either forms of (P)RR activates the tissue renin-angiotensin system (RAS) and for s(P)RR-bound prorenin, may also activate the circulating RAS. Also, when renin/prorenin binds to (P)RR, intracellular signaling pathways are triggered. *In vitro* experiments showed that the cell signalings are caused by both renin and prorenin in a manner independent of angiotensin[5-12] (Figure 2). Full-length and truncated (P)RR are capable of binding V-ATPase and are essential for V-ATPase assembly and function[13]. Extracellular domain of (P)RR binds Wnt receptor and serves as an adaptor for Wnt receptor and V-ATPase, and is now known to play important role in Wnt signaling, a key component of embryonic development[14-16].

Full-length (P)RR is known to be cleaved in the secretory pathway by proteases such as furin[3] and a disintegrin and metalloproteinase 19[17] to release s(P)RR into the circulation. Of the three different molecular forms, s(P)RR is the only molecule which is possible to be measured in human blood and urine samples. We have developed an s(P)RR enzyme-linked immunosorbent assay kit which allows quantification of s(P)RR in clinical settings[18]. The s(P)RR is now being measured in different pathological states. Recent study showed that increased plasma s(P)RR levels in pregnant women during the first trimester may predict the development of gestational diabetes mellitus (GDM) during the third trimester[19]. Plasma s(P)RR concentrations of neonates are higher than that of adults and the association between cord blood s(P)RR levels and small for gestational age (SGA) birth was shown[20], suggesting the involvement of (P)RR in embryo's growth.

In this review article, we make an attempt to figure out the possible pathophysiological roles of the (P)RR in pathogenesis of GDM and on embryo's growth.

(P)RR AND GLUCOSE INTORELANCE

Some data had shown the involvement of (P)RR on the pathogenesis of diabetes through angiotensin Ⅱ (AngⅡ) production. The activation of prorenin, without undergoing cleavage to renin was observed and AngⅡ contents increased in skeletal muscle tissues of fructose-induced rat models of insulin resistance[21]. Treatment with handle region peptide, inhibitory tool against prorenin binding (P)RR, markedly improved glucose tolerance, and this was associated with inhibition of nonproteolytic activation of prorenin by (P)RR and inhibition of increase in AngⅡ contents. Insulin resistance observed in obese Otsuka Long-Evans Tokushima Fatty rats was also associated with nonproteolytic activation of prorenin and increase in AngⅡ contents in the skeletal muscle and adipose tissues[22]. It has also been known that tissue RAS also exists in human pancreas and that it may directly affect β-cell function[23]. These findings indicate that (P)RR-bound prorenin may participate in the development of insulin resistance and β-cell function through tissue RAS activation.

Binding of (pro)renin to (P)RR also mediates Ang Ⅱ-independent signaling cascades. In vitro experiments

using the cells expressing the (P)RR showed the cell signaling caused by (pro)renin in an AngⅡ-independent manner. In the presence of angiotensin receptor antagonists, angiotensin converting enzyme inhibitors and/or renin inhibitors, the administration of prorenin/renin induced the activation of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2, leading to upregulation of transforming growth factor β1, independent of AngⅡ generation[6,10,11]. (P)RR also activates the MAPK p38 and subsequent phosphorylation of heat shock protein[5,9], and the phosphatidylinositol-3 kinase-p85 pathway[24]. Since activation of MAPK and transforming growth factor-β1-dependent pathways induced by insulin are known to contribute to the pathogenesis of insulin resistance[25,26] and MAPK p38 cascade is considered to regulate β-cell function[27-29], (P)RR-induced activation of these intracellular pathways may also contribute to the pathogenesis of glucose intolerance.

(P)RR also plays important role as V-ATPase associated protein[13]. It has been reported that a3 isoform of V-ATPase regulates the exocytosis of insulin from pancreatic β-cells[30]. It has been also shown that V-ATPase is involved in insulin-stimulated glucose transport in 3T3-F442A adipocytes[31]. From these data, we may can hypothesize that (P)RR contributes to development of diabetes also through V-ATPase-linked functions.

MATERNAL (P)RR

Human RAS physiologically undergoes drastic changes during pregnancy. Since ovary and maternal decidua produces renin, early increase in plasma renin activity is seen during pregnancy. Circulating estrogen released from the growing placenta increases angiotensinogen synthesis by the liver, leading to increase in serum AngⅡ and aldosterone levels. Previous study has demonstrated that fasting blood glucose (FBG) in pregnant women is inversely correlated with the plasma renin activity, whereas plasma aldosterone concentration showed a significant positive correlation with FBG during pregnancy. Moreover, PAC is significantly higher in pregnant women with GDM as compared to those with normal glucose tolerance during pregnancy[32]. These data support an idea that the RAS during pregnancy is involved in the pathogenesis of GDM.

Plasma prorenin/renin ratio differs in each pathophysiological state. In the plasma, prorenin levels mark approximately 10-fold higher than renin levels in normal physiological condition[33]. In the diabetic patients and in pregnant women, plasma prorenin levels increase up to 50 to 100-fold higher than that of renin[34]. Particularly, plasma prorenin concentrations can be used as an early predictor of microvascular complications in the diabetic patients[35]. High levels of prorenin are also observed in infants. In these states in which plasma prorenin/renin ratio increases, (P)RR may play the main role in their pathophysiology.

(P)RR is abundantly expressed in placenta[1]. As mentioned above, higher levels of plasma s(P)RR in an early stage of pregnancy were significantly associated with a higher possibility of developing GDM in a later stage in pregnacy[19]. Women in the highest plasma s(P)RR level quartile were 2.90-fold more likely to develop GDM than women in the lowest quartile. This data also supports the theory that (P)RR may be involved in the pathogenesis of GDM.

FETAL (P)RR

S(P)RR levels in umbilical cord blood were significantly higher than that of normal adult[18]. In addition, high plasma s(P)RR level in cord blood is associated with a lower SGA birth likelihood[20]. Developmental studies in Xenopus and Drosophila have revealed an essential role of (P)RR to promote the canonical and non-canonical Wnt signaling pathways[16]. Wnt proteins form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Now that it is indicated that (P)RR plays key role in Wnt signaling, these data indicate that (P)RR may be essential for embryo's growth.

(P)RR POSSIBLY CAUSES GDM AS A RESULT OF STIMULATING AN EMBRYO'S GROWTH

Fetuses of mothers who have diabetes are more likely to be large for gestational age (LGA) than fetuses of non-diabetic women. From the data that high s(P)RR level in cord blood associates with a lower SGA birth likelihood[20], it can be speculated that plasma s(P)RR levels are also high in LGA fetuses. If the inappropriate growth stimulation of embryo precede the onset of maternal glucose intolerance, fetal s(P)RR may be a factor which triggers the onset of GDM. As full-length (P)RR does, s(P)RR also activates prorenin[36], thereby leading to the activation of RAS, resulting in development of GDM. However, there are some limitations to this hypothesis (Figure 3).

First, the mechanism of placental transfer of s(P)RR is unclear. It has been known that molecules larger than 1000 molecular weight is incapable of passing from fetal circulation to maternal circulation[37]. The s(P)RR may be too large to pass through placenta, since its molecular weight is 28000[38]. However, upstream factors which regulates the expression of (P)RR may pass through placenta from fetus, leading to the augmentation of (P)RR also in maternal tissues.

Second, it is now considered, regarding mechanism of LGA birth in GDM, that maternal glucose passes through placenta and induces fetal hyperglycemia leading to increase in plasma insulin levels[39]. This theory conflicts with our hypothesis that stimulation of embryo's growth precedes the development of GDM. However, increase in fetal (P)RR expression, as a result of hyperinsulinemia, may affect maternal pathological condition, creating a vicious cycle and at least in part explain the

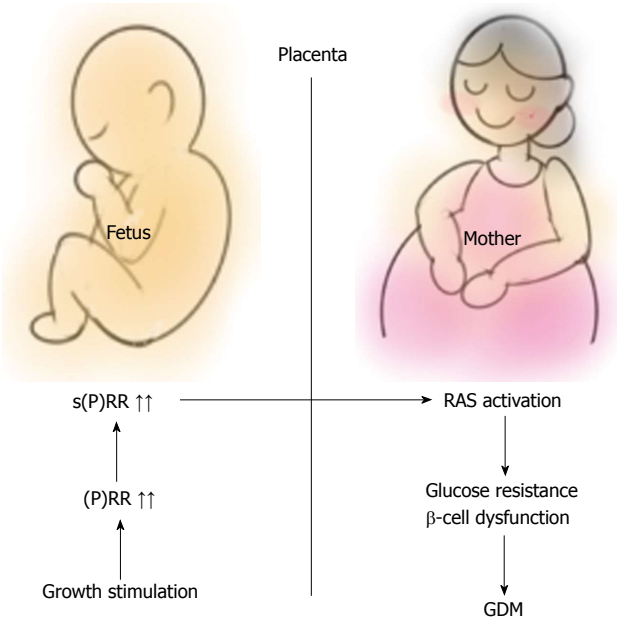

Figure 3 (Pro)renin receptor in the pathogenesis of gestational diabetes mellitus. (P)RR: (Pro)renin receptor; s(P)RR: Soluble (P)RR; RAS: Renin-angiotensin system; GDM: Gestational diabetes mellitus.

pathogenesis of GDM.

In conclusion, contribution of (P)RR to the pathogenesis of glucose intolerance has been speculated from previous studies. Although there is a lack of direct evidence, we highlighted the possibility of (P)RR-mediated fetal-maternal interaction as a pathogenesis of GDM. Measurement of maternal and cord blood s(P)RR levels in GDM patients at delivery will be needed to consolidate the theory. Also, time-course analysis of maternal and fetal s(P)RR in animal GDM model may provide evidences which may support pathogenetic role of (P)RR-mediated fetal-maternal interaction. Further investigations are needed, but this novel hypothesis may lead us to new diagnostic and therapeutic strategies for GDM.

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P- Reviewer: Efstathiou S, Zhao D **S- Editor**: Tian YL
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