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Long-term effects of metformin use in gestational diabetes mellitus on offspring health

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Abstract

Metformin is the first-line drug for the treatment of type 2 diabetes mellitus, but its role in gestational diabetes mellitus (GDM) management is not clear. Recent evidence suggests a certain beneficial effect of metformin in the treatment of GDM, but a high treatment failure rate leads to the initiation of additional medications, such as insulin. Moreover, since metformin crosses the placental barrier and reaches a significant level in the fetus, it is likely to influence the fetal metabolic milieu. The evidence indicates the long-term safety in children exposed to metformin in utero except for mild adverse anthropometric profiles. Diligent follow-up of metformin-exposed offspring is warranted from the clinician's point of view.

Key Words: Anthropometry; Fetal; Gestational diabetes mellitus; Long-term; Metformin; Offspring

Core Tip: The use of metformin in mild-to-moderate gestational diabetes mellitus may confer certain advantages. Since metformin reaches almost a similar serum level in the fetus, it is likely to influence the fetal metabolic environment. Limited long-term data suggest that metformin-exposed children have mild adverse anthropometric profiles. However, the clinical significance and effect on cardiometabolic health have yet to be determined.

Citation: Roy A, Sahoo J. Long-term effects of metformin use in gestational diabetes mellitus

INTRODUCTION

Metformin is a biguanide compound used as a first-line drug for the treatment of type 2 diabetes mellitus. However, the use of metformin in gestational diabetes mellitus (GDM) is still debated. Currently, the American Diabetes Association does not recommend metformin as a first-line therapy in GDM patients, mainly due to the absence of long-term safety data of metformin exposure in utero, and many patients additionally require insulin for the control of glycemia[1]. However, the National Institute for Health and Care Excellence Guideline of the United Kingdom recommends the use of metformin in GDM patients when diet and exercise for 1-2 wk alone fail to control hyperglycemia[2]. This is particularly recommended when fasting plasma glucose (FPG) is less than 126 mg/dL (7 mmol/L). Of note, this guideline mentions the use of metformin as off-label. This guideline also recommends using insulin with or without metformin in GDM mothers in whom FPG is greater than 126 mg/dL (7 mmol/L).

Metformin predominantly acts by decreasing hepatic glucose output by decreasing gluconeogenesis. The principal molecular mechanisms responsible are activation of adenosine monophosphate-dependent kinase and inhibition of complex I of the respiratory chain in mitochondria[3]. However, it was recently shown that metformin also decreases glycerol- and lactate-dependent gluconeogenesis by inhibiting glycerol-3-phosphate dehydrogenase enzyme[3]. Additionally, metformin acts through the intestine by modulating the gut microbiome[4], increasing glucagon-like peptide 1 secretion[5] and altering bile acid metabolism[6].

The proposed rationale of the use of metformin in patients with GDM, gestational obesity and pregnancy with polycystic ovary syndrome (PCOS) lies in its beneficial effects demonstrated in several clinical trials. Metformin was found to result in similar neonatal outcomes as insulin in GDM patients without any increase in serious adverse events in earlier studies[7,8]. More women preferred metformin over insulin during clinical trials. A recently concluded large randomized controlled trial (RCT)[9] (MiTy trial) showed multiple benefits of metformin treatment compared to placebo in GDM patients. The metformin-treated group had better glycemic control with reduced insulin requirements and less weight gain of the mother during pregnancy. Moreover, metformin-exposed infants were lighter with lower adiposity and a higher risk of being small for gestational age. A previous meta-analysis[8] reported that metformin treatment was associated with a decreased risk of neonatal hypoglycemia and large for gestational age newborns with less weight gain of the mother during pregnancy. Furthermore, metformin treatment was not associated with an increased risk of preterm delivery, perinatal mortality, small for gestational age infants, and cesarean section. Thus, it is possible that metformin might play a role in the management of GDM, gestational obesity, and pregnant women with PCOS.

There is evidence that metformin crosses the placental barrier and reaches a similar serum level in the fetus as in the mother[10], thus implying its role in the modification of the metabolic milieu of the offspring. Thus, long-term data on offspring outcomes remain an important consideration before choosing metformin for use during pregnancy. Hence, we provide a short summary and discuss future needs in this context.

LONG-TERM EFFECTS OF METFORMIN ON OFFSPRING OF MOTHERS WITH GESTATIONAL DIABETES

Effect on anthropometry and metabolic parameters of offspring

The metformin in a gestational diabetes follow-up (MiG-TOFU) cohort provided invaluable insights into offspring health[11]. The first 2-year follow-up data showed that children exposed to metformin during pregnancy had higher subcutaneous fat measurements as measured by mid-upper arm circumferences, biceps and subscapular skinfold thickness. However, they had similar total fat mass and percentage of body fat as measured by bioimpedance analysis and dual-energy X-ray absorptiometry

when compared to the insulin-exposed counterparts, as shown in Table 1. Although an ethnic difference in the prevalence of higher visceral adiposity was suggested, particularly in Indian boys at the 2-year follow-up of the MiG cohort[12], the specific effect of metformin is not known at present. The MiG cohort study was further extended to 7 years in the Adelaide cohort and 9 years in the Auckland cohort[13]. In the Adelaide cohort, there was no difference in outcomes in the offspring between the metformin and insulin treatment groups. However, the metformin-exposed group in the Auckland cohort had significantly higher weight, arm circumference, waist circumference (WC), and waist-to-height ratio. The abdominal fat components, including visceral adipose tissue, subcutaneous adipose tissue and liver fat measured by magnetic resonance imaging, were similar in the metformin group as the insulin-exposed group[13]. However, there was a trend toward a higher fat mass per volume in the metformin-exposed group. Different metabolic parameters were also found to be similar between the two groups (FPG, glycosylated hemoglobin, triglyceride, cholesterol, insulin, liver enzymes, leptin, adiponectin and biochemical markers of insulin resistance). Whether this finding is explained by the higher body mass index (BMI) of the mothers randomized to the metformin arm at the time of recruitment or the decreased nutritional intake during the later part of pregnancy is not clear.

Ijäs *et al*[14] reported a higher body weight at 12 mo and higher height and body weight at 18 mo in metformin-exposed offspring than in insulin-exposed offspring. Metformin exposure and pre-pregnancy BMI of the mother were identified as predictors of higher body weight at 18 mo during the follow-up of the offspring. However, the BMI and the percentage of overweight and obese children were not different between these two treatment groups. This study did not report any difference in terms of subcutaneous or visceral adiposity. Another observational study performed in school children from New Zealand did not find any difference in weight-for-height z-scores in the offspring of metformin- *vs* insulin-treated GDM mothers[15]. This study also did not find any difference in terms of the likelihood of having a weight for height percentile greater than the 85th percentile in the children of metformin-exposed mothers as compared to insulin treated counterparts. A recent study from India reported nine years of follow-up data of the offspring of mothers who were randomized either to metformin or glibenclamide during their GDM management[16]. They found no difference in BMI, WC or visceral fat distribution between the treatment groups. All metabolic parameters were comparable between the two groups except for a mild increase in the triglyceride levels in the metformin group. Similarly, there was no difference in the mean systolic or diastolic blood pressure (BP) among the offspring of metformin- or insulin-treated mothers in the MiG trial at the median follow-up of 29 mo of age[17].

A meta-analysis[18] performed on 684 children concluded that metformin-exposed children were heavier [standardized mean difference 0.26, 95%CI (0.11–0.4)] (heterogeneity $I^2 = 0\%$)]. Other measurements, such as body composition and height, were not different in the metformin-exposed group compared to those in the insulin/placebo group. The heavier weight of offspring during follow-up in the metformin-exposed group may reflect the effect of lower birth weight. Indeed, a recent meta-analysis has shown that metformin-exposed neonates were lighter than both glibenclamide- and insulin-exposed neonates[19]. There is evidence from animal studies that metformin-exposed offspring are born lighter and later gain more weight when fed high-fat diets [20].

Effect on other offspring parameters

Apart from anthropometric data, few studies have explored other aspects of long-term health in the offspring of metformin-exposed mothers. The psychosocial and behavioral indices were similar between the treatment groups in the study by Landi *et al*[15]. Wouldes *et al*[21] found no difference in terms of neurodevelopmental skills between the groups at the two-year follow-up of the MiG trial. Another study examined the testicular size of offspring born to either metformin- or insulin-treated GDM mothers[22]. They did not find any difference in testicular size measured by orchidometer or testicular ultrasound at the mean age of 60 mo. However, the sample size was small, and it would be interesting to note the difference in testicular size and resultant impact on gonadal function and fertility after the onset of puberty rather than at five years of age.

Long-term effect of metformin on offspring in reference to mothers with gestational obesity and PCOS

The other two clinical conditions where metformin can be used during pregnancy are

Table 1 Summary of the selected studies on the long-term effect of metformin on the anthropometry and metabolic parameters of the offspring

Ref.	Year	Country	Follow-up timing	Main outcomes
Rowan *et al*[11]	2011	Australia and New Zealand	2 yr	Metformin exposed children had (1) Larger mid-upper arm circumferences, biceps and subscapular skinfold thickness; and (2) Total fat mass and percentage body fat were similar to insulin group
Rowan *et al*[13]	2018	Australia and New Zealand	7 yr and 9 yr in Adelaide and Auckland cohort respectively	No difference in the metformin-exposed children and insulin-treated mothers in Adelaide cohort. In Auckland cohort: (1) Metformin-exposed children had larger weight, arm and waist circumferences, and waist: Height ratio; (2) Similar body fat percentage between two treatment groups; and (3) Visceral adipose tissue, abdominal subcutaneous adipose tissue and liver fat were similar in metformin exposed group in comparison to insulin treatment
Ijäs *et al* [14]	2014	Finland	mo	(1) Children exposed to metformin were significantly heavier at the age of 12 mo; and (2) Metformin exposed offspring were taller and heavier at the age of 18 mo
Landi *et al*[15]	2019	New Zealand	4 yr	No significant differences in weight, weight for height, or body mass index in children of insulin versus metformin exposed mothers
Paul *et al*[16]	2020	India	9 yr	(1) No difference in weight, body mass index, waist circumference, body fat percentages in between metformin and glibenclamide exposed children; and (2) Similar metabolic profile between two groups except mild elevation of serum triglyceride in the metformin group

gestational obesity and PCOS. However, the data regarding the long-term outcome in such settings are very sparse[23-27]. A recently concluded obese pregnant woman offspring follow-up study (77 metformin-exposed children aged 3.9 ± 1.0 years) noted similar peripheral arterial BP, arterial stiffness, and metabolic parameters (lipid profile, leptin and adiponectin) between the metformin and placebo groups[23]. The body composition was similar between the two groups except for lower gluteal and triceps circumferences in the metformin group. Interestingly, metformin-exposed children showed lower central cardiovascular hemodynamic indices and diastolic indices. Further insight into the long-term outcome associated with metformin use in women with gestational obesity will be possible when the follow-up data of two important RCTs become available (EMPOWaR and GRoW trials) in the future.

The other context is the use of metformin for PCOS. A small follow-up study did not find any difference in body composition between metformin- and placebo-exposed children at 8 years of age, but a higher FPG and systolic BP were noted in the metformin group[24]. In one of the longest follow-up RCTs, it was found that markers of obesity, such as BMI, BMI-z scores, waist-to-height ratio, and WC, were higher in metformin-exposed children than in the children in the placebo group[25]. The obese phenotype was evident by the age of 4 years (67% metformin-exposed children were either overweight or obese by 4 years of age). However, the biochemical measures of metabolic syndrome, which usually develops later in life, were similar between the groups. Combined follow-up of this RCT and its pilot study also showed similar trends of increased BMI and overweight/obese percentage among metformin-exposed children born from a PCOS pregnancy[26]. However, metformin exposure does not affect the cognitive outcome in children born to mothers with PCOS[27]. Thus, it is evident that even in the context of gestational obesity and PCOS, metformin exposure alters both anthropometric and metabolic profiles of the offspring.

CONCLUSION

To date, the evidence has indicated the long-term safety of exposure to metformin in utero except for mild adverse metabolic profiles. However, the data are limited in quantity and quality. Several questions remain to be clarified further about the long-term safety in offspring exposed to metformin in utero. First, there were not enough studies reporting long-term data. Moreover, long-term studies are prone to high dropout rates. Second, the impact of alterations in anthropometric data on cardiometabolic outcomes must be determined further in future studies. Third, whether the effect of metformin will continue until adulthood is an important point to explore. Fourth, whether metformin has a differential impact on offspring health based on ethnicity, particularly in low-income countries, needs to be explored in the future. Finally, further basic research is needed to identify and characterize the incongruity between animal studies and human follow-up study outcomes.

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REVIEW

Organophosphate pesticides and new-onset diabetes mellitus: From molecular mechanisms to a possible therapeutic perspective

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Abstract

Organophosphate is a commonly used pesticide in the agricultural sector. The main action of organophosphate focuses on acetylcholinesterase inhibition, and it therefore contributes to acute cholinergic crisis, intermediate syndrome and delayed neurotoxicity. From sporadic case series to epidemiologic studies, organophosphate has been linked to hyperglycemia and the occurrence of new-onset diabetes mellitus. Organophosphate-mediated direct damage to pancreatic

beta cells, insulin resistance related to systemic inflammation and excessive hepatic gluconeogenesis and polymorphisms of the enzyme governing organophosphate elimination are all possible contributors to the development of new-onset diabetes mellitus. To date, a preventive strategy for organophosphate-mediated new-onset diabetes mellitus is still lacking. However, lowering reactive oxygen species levels may be a practical method to reduce the risk of developing hyperglycemia.

Key Words: Organophosphate; Pesticide; New-onset diabetes mellitus; Mechanism; Reactive oxygen species

Core Tip: Organophosphate may induce acute hyperglycemia by damaging pancreatic cells and result in new-onset diabetes mellitus after chronic exposure to organophosphate compounds. Organophosphate-mediated new-onset diabetes mellitus might be mediated by a polymorphism of paraoxonase-1, which is associated with organophosphate elimination in hepatocytes. Pancreatic beta cell damage, excessive gluconeogenesis, hepatic steatosis, systemic inflammation and possibly sarcopenia all contribute to insulin resistance and therefore hyperglycemia.

INTRODUCTION

Organophosphate is a commonly used pesticide in the agricultural sector because of its bioavailability. The main action of the organophosphate focuses on acetylcholinesterase inhibition. Because of its wide use, intoxication of organophosphate has been commonly encountered by physicians. Intoxication can be divided into acute cholinergic crisis, intermediate syndrome and delayed neuropathy[1]. Among the complications induced by organophosphates, diabetes mellitus is a common yet often overlooked metabolic complication. The aim of this review is to analyze the molecular pathogenesis mechanisms of new-onset diabetes mellitus after organophosphate exposure.

ORGANOPHOSPHATE TOXICITY: ACUTE CHOLINERGIC CRISIS AND CHRONIC OXIDATIVE STRESS GENERATION

The main action of organophosphate is to inhibit acetylcholinesterase within the nervous system, and therefore, acetylcholine overactivity exists within the synapse and neuromuscular junction[2]. Neurological manifestations are the cardinal symptoms of organophosphate intoxication through the activation of muscarinic receptors and include myosis, excessive secretions, seizures, severe muscle paralysis, cardiorespiratory depression and even death in organophosphate overdose patients [3]. The hydrophobic character of organophosphate leads to its accumulation in adipose tissue, and therefore, intermediate syndrome with delayed neurologic injury might occur through the generation of oxidative stress. Gultekin *et al*[4] demonstrated that organophosphate treatment could activate lipid peroxidase and therefore generate reactive oxygen species (ROS) by exhausting glutathione and superoxide dismutase in a dose-dependent manner. Similar oxidative stress with excessive acetylcholinesterase activity has been reported in workers with chronic exposure to organophosphate[5]. Apart from neurotoxicity, accumulation within different tissues could cause different end-organ damage in the chronic phase. The mitogen-activated protein kinase

(MAPK) signaling pathway could activate associated kinases, such as extracellular responsive kinases, c-Jun N-terminal kinase (JNK) and p38 MAPK, which could worsen downstream apoptosis[6]. The main contributor to MAPK signaling from organophosphates is mediated by oxidative stress. From *in vitro* studies, the administration of organophosphate could activate the expression of quinone oxidoreductase-1, heme oxygenase 1, paraoxonase-1, catalase or superoxide dismutase in blood mesenchymal stem cells[7] or human umbilical vein endothelial cells[8]. Therefore, distant organ damage should arouse concern in chronic organophosphate-intoxicated subjects.

CLINICAL STUDIES OF NEW-ONSET DIABETES MELLITUS AFTER ORGANOPHOSPHATE EXPOSURE

Previous studies revealed that organophosphate exposure could increase the risk of new-onset diabetes mellitus (Tables 1 and 2). Moore and James[9] first noticed that acute organophosphate ingestion was associated with hyperglycemia, and hyperglycemia required insulin intervention for blood sugar control (Table 1). Serial studies also demonstrated that organophosphate-mediated acute pancreatic injury might induce hyperglycemia[10,11]. In 2008, Montgomery *et al*[12] provided epidemiologic data to link chronic exposure to organophosphate with diabetes mellitus (Table 2). Within the 5-year follow-up, the incidence of diabetes mellitus increased in organophosphate users. The study conducted by Liu *et al*[13] demonstrated that acute exposure to organophosphate led to hyperglycemia, but the effect on the development of diabetes mellitus was only marginal. In a meta-analysis study conducted by Lakshmi *et al*[14], hyperglycemia was common. A recent study by Panda *et al*[15] demonstrated that organophosphate exposure was associated with higher insulin resistance and higher plasma glycated hemoglobin levels. From the clinical study, acute organophosphate exposure was associated with hyperglycemia and then regressed after atropine treatment. From the study published by Leonel Javeres *et al* [16], red blood cell acetylcholinesterase activity decreased within the organophosphate exposure group, and the plasma concentrations of lipase/amylase and insulin increased in the organophosphate-exposed group. Such evidence demonstrated the effect of organophosphate on insulin resistance and direct damage to pancreatic cells in clinical investigations.

Clinical studies have shown that acute hyperglycemia develops in acute organophosphate-intoxicated subjects and that such hyperglycemia is associated with poor clinical patient outcomes. However, hyperglycemic status was mostly observed in animals with chronic or subchronic exposure[17,18]. Several *in vivo* studies demonstrated the acute effect of organophosphate on the variation of blood sugar. Rodrigues *et al*[19] reported variations in blood sugar after acute organophosphate exposure. For rats receiving a single intraperitoneal injection of malathion, blood glucose increased within 2 h, followed by hypoglycemia 8 h after injection[19]. In brain tissue, organophosphates can decrease the storage of glycogen within the brain by activating glycogenolytic enzymes such as glycogen phosphorylase and phosphoglucomutase[20]. Glycolytic enzymes, such as phosphofructokinase and hexokinase, might decrease in the acute exposure of organophosphate[21]. Collectively, these mechanisms could explain the occurrence of acute hyperglycemia following organophosphate exposure.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANOPHOSPHATE EXPOSURE: DYSFUNCTION OF PANCREATIC BETA CELLS

Nagaraju and Rajini[22] reported that rats receiving chronic organophosphate had higher insulin secretion from pancreatic islet cells and associated pancreatic hypertrophy. Insulin plays an important role in activating glucose transporter 9-mediated glucose transport into cells. Therefore, the regulation of insulin secretion is important in mediating the plasma concentration of glucose. Acetylcholinesterase lies within the pancreas either within acinar cells or insulin-secreting beta cells[23,24]. In insulin-secreting beta cells, acetylcholine binds to the muscarinic receptors of beta cells and then increases the cytosolic calcium concentration and enhances the efficiency of calcium-mediated exocytosis, which activates insulin-secreting activity[24]. Acetylcholinesterase also occurred within the alpha cells of the pancreas. Alpha cells

Table 1 Published human studies on the association between acute organophosphate exposure and the development of new-onset diabetes mellitus

Ref.	Area	Pesticide	Exposure	Sample size	Association
Moore and James[9], 1980	Australia	Coumaphos	Acute	1	Hyperglycemia
Hui[10], 1983	Hong Kong	Organophosphate	Acute	2	Hyperglycemia
Weizman and Sofer [11], 1992	Israel	Organophosphate and carbamate	Acute	17	Hyperglycemia in 29.4% of patients
Yurumez *et al*[98], 2007	Turkey	Organophosphate	Acute	220	Hyperglycemia in 67.7% of patients
Liu *et al*[13], 2014	Taiwan	Organophosphate	Acute	118	Hyperglycemia after poisoning was not associated with higher mortality
Moon *et al*[99], 2016	South Korea	Organophosphate	Acute	184	Hyperglycemia after poisoning was associated with higher mortality

Table 2 Published human studies on the association between chronic organophosphate exposure and the development of new-onset diabetes mellitus

Ref.	Area	Pesticide	Exposure	Sample size	Association
Montgomery *et al* [12], 2008	United States	Organophosphate and organochlorine	Chronic	33457	Positive association with diabetes
Raafat *et al*[100], 2012	Egypt	Malathion	Chronic	98	Positive associations among blood malathion concentration, waist circumference and insulin resistance
Velmurugan *et al* [30], 2017	India	Organophosphate	Chronic	3080	Positive association between blood organophosphate residues and glycated hemoglobin levels
Velmurugan *et al* [90], 2020	India	Organophosphate and arsenic	Chronic	865	Positive associations of organophosphate and arsenic with diabetes, prediabetes and atherosclerosis

stimulate insulin secretion in a paracrine manner within the pancreas[25]. Bendayan and Gisiger[23] also reported that acetylcholinesterase existed within acinar cells. Acinar cells are commonly regarded as governing lipase, but insulin secretion ability has been noted in several human studies beyond alpha and beta islet cells[26]. Case series studies showed that organophosphate overdose could induce pancreatitis and elevation of serum amylase[27]. Such clinical studies have provided evidence of organophosphate-mediated pancreatic damage. In addition, the acetylcholinergic receptor also governs the viability of pancreatic cells. The study conducted by Pfitzinger *et al*[28] demonstrated that cholinergic activation slowed the progression of pancreatic cancer. On the other hand, Zhang *et al*[29] presented evidence in a type I diabetes mellitus animal model mediated by streptozotocin that an acetylcholin-esterase inhibitor protected pancreatic beta cells against apoptosis. Therefore, organo-phosphates might disrupt insulin secretion directly by dysregulating acetylcholin-esterase activity (Figure 1).

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: DYSFUNCTION OF GLUCONEOGENESIS

Organophosphate-mediated gluconeogenesis by disrupted lipolysis

The pathogenesis of diabetes mellitus involves impaired regulation of hepatic glucon-eogenesis. Hypersensitive glucose production in response to gluconeogenic stimuli poses organophosphate exposure as a risk factor for prediabetes[30,31]. As organo-phosphates are ingested *via* the intestine, the conversion of organophosphates by cytochrome 450 enhances cholinergic inhibition up to 70%[32]. As organophosphate accumulates within hepatocytes, the activation of adenylyl cyclase produces excessive cyclic adenosine monophosphate[31], which increases hepatic glucose production and
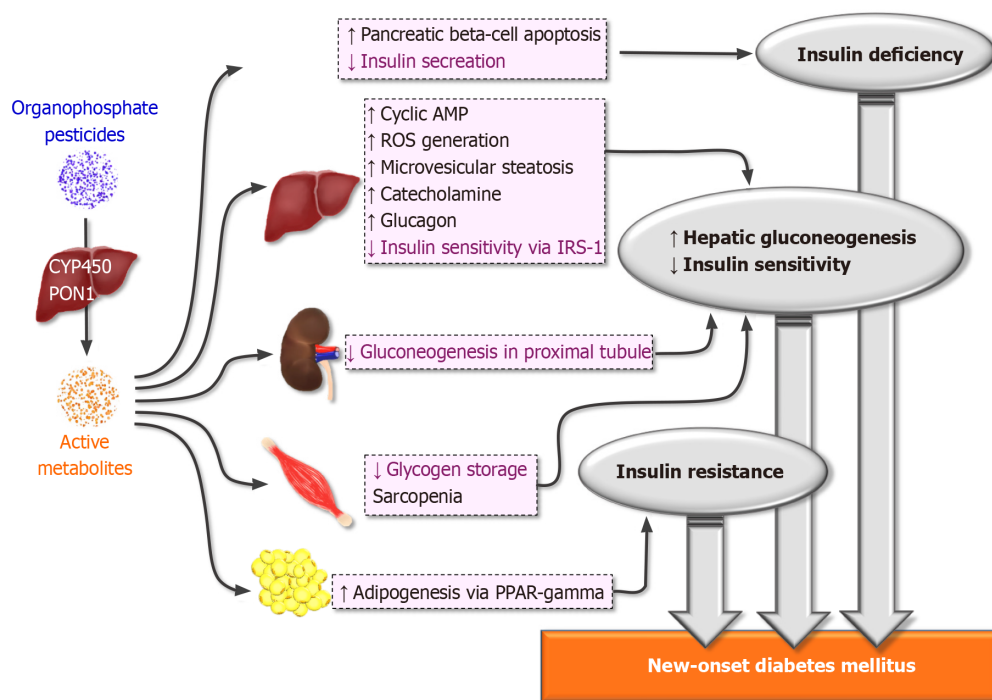
Figure 1 Postulated molecular mechanisms of new-onset diabetes mellitus after organophosphate pesticide exposure. Organophosphate pesticides are metabolized in the liver to toxic derivatives *via* cytochrome P450 *via* a first-pass effect. Paroxonase isoform 1 serves as the enzyme handling hydroxylation and cleavage of the toxic form. The active metabolite might induce new-onset diabetes mellitus *via* pancreatic beta cell damage and disturb the homeostasis of gluconeogenesis and insulin sensitivity. Organophosphates could directly induce apoptosis of pancreatic beta cells by activating nuclear factor-κappa beta, and therefore, insulin secretion may be hampered. Beyond pancreatic cells, gluconeogenesis within the liver could be activated by reactive oxidative species generation and inflammation induced by microvesicular steatosis, enhanced cyclic adenosine monophosphate generation, excessive catecholamine and abated insulin sensitivity of hepatocytes *via* insulin receptor substrate-1. Distal organ damage by organophosphates may also disturb the homeostasis of gluconeogenesis. In organophosphate-related acute kidney injury, gluconeogenesis within the proximal tubules is disturbed. Decreased proximal gluconeogenesis exacerbates excessive hepatic gluconeogenesis. In skeletal muscle, sarcopenia mediated by intermediate syndrome might reduce glycogen storage within skeletal muscle, which may induce hyperglycemia. Organophosphates also deposit within adipose tissue and therefore exacerbate adipogenesis by activating peroxisome proliferator-activated receptor-gamma. Excessive adipose tissue might enhance insulin resistance and further hasten the development of new-onset diabetes mellitus. CYP450: Cytochrome P450; PON1: Paroxonase isoform 1; ROS: Reactive oxidative species; AMP: Adenosine monophosphate; IRS-1: Insulin receptor substrate-1; PPAR: Peroxisome proliferator-activated receptor.

therefore increases body weight along with adipose tissue[33]. In a study conducted by Velmurugan *et al*[30], acetic acid increased hepatic glucose-6 phosphate and citric acid production after inducing inflammation. Apart from activation of cyclic adenosine monophosphate, the organophosphate itself also increases oxidative stress within hepatocytes by exhausting enzymes that reverse oxidative stress[34], and such oxidative stress may disrupt membranous lipids by activating lipid peroxidation[35]. Hepatic injury also occurs in organophosphate intoxication, and therefore, sequential sinusoidal dilatation and microvesicular steatosis impair glycogen synthesis[36]. Insulin mediates the suppression of adipose lipolysis physiologically and therefore downregulates gluconeogenesis[37]. Ince *et al*[38] demonstrated that lipid metabolism was disturbed in organophosphate-treated mice, with excessive end-products of lipid peroxidation. As excessive acetylcholinesterase leads to subjects having chronic hypercholinergic status, dietary habits are altered. From the study by Slotkin *et al*[39], neonatal rats exposed to organophosphate had hyperactive acetylcholine function within the neuron body, and such activity could be ameliorated only by a high-fat diet.

Organophosphate-mediated gluconeogenesis mediated by impaired glycogen storage

Under hyperglycemic conditions, glycogen storage could lower circulating glucose and enhance the anabolism process rather than catabolism. Glycogen phosphorylase, which counteracts glycogen storage, is activated by organophosphates[40]. Dichlorvos, as an example, increased the messenger ribonucleic acid expression of glycogen phosphorylase and decreased glycogen storage[41]. However, the different organophosphates had diverse actions on glycogen-storing proteins. Malathion, while activating the gluconeogenesis process, decreased glycogen phosphorylase but resulted in compensatory hepatomegaly[42,43]. The modulation of organophosphate

on glycogen storage could contribute to the gluconeogenesis process.

Organophosphate-mediated gluconeogenesis mediated by altered hormone regulation

Glucagon and catecholamine are the major hormones regulating gluconeogenesis[37]. Glucagon and catecholamine could directly enhance hepatic gluconeogenesis by activating cyclic adenosine monophosphate *via* phosphorylation of protein kinase A activity[44] and bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFK2/FBPase-2)[45]. Glucagon activity also decreased glycogen storage by coupling with the inhibitory G protein[46]. Catecholamine, on the other hand, also activates gluconeogenesis *via* cyclic adenosine monophosphate activity[47]. Stress-associated catecholamine release can increase the gluconeogenesis process and therefore insulin resistance, and organophosphate itself activates catecholamine release after inhibiting acetylcholinesterase activity[42]. Organophosphate could activate catecholamine release within neurons, and unbalanced catecholamine release might prolong the gluconeogenesis process.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: INSULIN RESISTANCE MEDIATED BY INFLAMMATION OR DYNAMIC CHANGES IN THE MICROBIOTA

Insulin resistance, excessive gluconeogenesis and insufficient glucose uptake in the presence of insulin place subjects as hyperglycemia status and therefore invoke sequential adipose tissue formation. Physiologically, insulin activates the insulin receptor by tyrosine phosphorylation of insulin receptor substrate-1[48], and serine phosphorylation inhibits the insulin receptor and offsets insulin activity[49]. Insulin resistance is common in chronic organophosphate exposure subjects. From the *in vivo* study conducted by Nagaraju and Rajini[22], insulin hypertrophy and the increased secretion of insulin were accompanied by circulating insulin-like growth factor 1, free fatty acids, corticosterone, and paraoxonase activity. As organophosphate increases excessive cholinergic activity, insulin resistance is associated with systemic inflammation. From the study reported by Liang *et al*[50], organophosphates could induce an increase in body weight in experimental mice treated with a high-fat diet. In organophosphate mice treated with a high-fat diet, systemic inflammation mediated by lipopolysaccharide might occur. Systemic inflammation mediated by the intestinal barrier might activate systemic inflammation. In addition, the lipid peroxidation end product malondialdehyde (MDA) increased in organophosphate-treated rats, and the oxidative end product was associated with a higher level of inflammation[38].

Chronic exposure to organophosphate could directly enhance systemic inflammation. In a study by Ince *et al*[38], organophosphate-treated rats had higher proinflammatory cytokines, such as interferon gamma, interleukin 1 beta, tumor necrosis factor alpha, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), than control rats. Hepatocytes are the major cells confronting proinflammatory cytokines. As NF-κB is activated by inflammatory cytokines, IκB kinase-β might be activated and therefore hamper the downstream action of insulin[51]. In addition to IκB kinase-β, JNK signaling was also activated in organophosphate intoxication. When endoplasmic reticulum stress is enhanced after organophosphate-mediated excessive oxidative stress, JNK is activated under conditions of proinflammatory cytokine release *via* NF-κB[52]. Based on the evidence above, organophosphates might induce inflammation and produce proinflammatory cytokines mediating insulin resistance.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: INSULIN RESISTANCE MEDIATED BY ROS

Excessive oxidative stress is associated with insulin resistance. Polyunsaturated fatty acids are the main source of oxidative stress. Low concentrations of ROS mediate the proliferative signals of insulin by phosphatidylinositol 3-kinase and protein kinase B [53]. In acute or chronic organophosphate intoxication, ROS regeneration is common as the exhaustion of endogenous antioxidant species occurs. The MDA level and superoxide dismutase increased in organophosphate intoxication, and reduced

glutathione was depleted[5]. Possamai *et al*[54] showed that both acute and chronic exposure to malathion could generate ROS within the kidney and brain acutely and liver and skeletal muscle chronically. The study performed by Aly *et al*[55] also demonstrated that the liver serves as the reservoir in chronic organophosphate exposure with the generation of ROS. To enhance the elimination of ROS, adenosine triphosphate is generated from the activated gluconeogenesis process within the liver [56]. In addition, ROS also directly disturb insulin receptor signaling. Morino *et al*[57] demonstrated that ROS activated serine residues on insulin receptor substrate 1 and therefore inhibited glucose transporter type 4. From the aspect above, the ROS generated by organophosphate could disturb insulin signaling and therefore worsen insulin resistance.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: DYSFUNCTION OF PEROXISOME PROLIFE-RATOR-ACTIVATED RECEPTOR GAMMA

Peroxisome proliferator-activated receptor (PPAR) is a transcriptional receptor within the nucleus, and its main action governs the proliferation of peroxisomes within the nucleus. PPARs regulate the metabolism of carbohydrates, lipids and proteins along with insulin sensitivity. The role of organophosphates in lipid metabolism has been demonstrated. Since organophosphate is a highly fat-soluble component, accumulation within adipose tissue could prolong its toxicity and generate oxidative stress within adipose tissue[58]. Smith *et al*[59] demonstrated that diazinon induces adipogenesis within preadipocytes by activating PPAR gamma receptors along with the transcription factor CCAAT-enhancer-binding protein α (C/EBPα).

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AGONIST AND DIPEPTIDYL PEPTIDASE-4 INHIBITOR

Incretin secreted from the intestine is important for insulin secretion. As carbohydrates enter the duodenum, the K cells within the duodenum secrete glucose-dependent insulinotropic polypeptides into the brain *via* the vagus nerve. Activated vagal tone enhances acetylcholine release to M cells within the distal ileum and therefore increases glucagon-like peptide 1 (GLP-1). GLP-1 could therefore increase insulin release and lower blood glucose. Organophosphates might increase the acetylcholine concentration within the neural cleft and therefore downregulate muscarinic receptors. Downregulated muscarinic receptors attenuate GLP-1 release and therefore further insulin release[60]. From the study by Rathis *et al*[61], the GLP-1 response was attenuated in subjects with acute exposure to organophosphate with atropine treatment. Chronic exposure to organophosphate might downregulate the incretin-mediated glucose-lowering effect.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: RENAL HANDLING OF GLUCOSE

From clinical observations, victims of organophosphate exposure had transient glycosuria, which was relevant to euglycemia[62]. Based on the evidence, acute tubular necrosis might be noticed in organophosphate intoxication subjects. From the study reported by Kaya *et al*[63], acute organophosphate intoxication could mediate the vacuolization of tubular epithelial cells and tubular structure approaching atrophy within the proximal tubules. The oxidative stress mediated by organophosphates worsened proximal tubular damage in an *in vitro* study performed by Poovala *et al* [64]. The activation of the MAPK signaling pathway within nephron precursor cells demonstrated direct nephrotoxicity after activating JNK and caspase-3[65]. Proximal tubular cells primarily serve as gluconeogenic cells through the utilization of adenosine triphosphate, and therefore, damaged proximal tubules might impair endogenous gluconeogenesis[66] and are associated with higher mortality and the need for dialysis in critically ill patients[67,68], including organophosphate in-

toxication subjects[69]. Since acute kidney injury and stress-mediated inflammation might contribute to insulin resistance and new-onset diabetes mellitus[70], preserving kidney function during acute kidney injury status through organophosphate intoxication should be important in managing these patients to hamper the development of diabetes mellitus.

MECHANISMS OF NEW-ONSET DIABETES MELLITUS AFTER ORGANO-PHOSPHATE EXPOSURE: INTERMEDIATE SYNDROME

As described in the previous sections, the major clinical manifestation of cholinergic crisis was the overactivation of the parasympathetic tone with tachycardia, myosis or neurologic complications such as seizures. From a previous study by Liu *et al*[13], acute organophosphate intoxication was minimally predictive of new-onset diabetes mellitus. Intermediate syndrome by organophosphate could induce myopathy, especially in the proximal skeletal muscle and respiratory muscle[71]. Although the direct mechanism is still unknown, necrosis of skeletal muscle and associated myopathy might be an entity in chronic organophosphate intoxication subjects[72]. In addition, organophosphate-mediated peripheral motor neuropathy is accompanied by weakness after acute intoxication[73]. From a review of the literature, a correlation between organophosphate intoxication and sarcopenia was rare. However, in patients with diabetic neuropathy, sarcopenia was more obvious than in those without neuropathy[74]. Persistent muscle weakness in intermediate syndrome might lead to sarcopenic status in organophosphate patients, and sarcopenia alone might enhance the risk of developing diabetes mellitus. In the study by Hong *et al*[75], skeletal muscle mass was negatively associated with the development of type 2 diabetes mellitus. Since skeletal muscle serves as the pool of glucose mediated by insulin, the decreased skeletal mass would reduce glucose disposal and therefore worsen the inflammation of skeletal muscle and insulin resistance[76].

POSSIBLE THERAPEUTIC PERSPECTIVE IN PREVENTING ORGANO-PHOSPHATE-MEDIATED NEW-ONSET DIABETES MELLITUS

In acute organophosphate intoxication, the application of atropine is the mandatory therapeutic strategy in treating cholinergic crises. The association between atropine and insulin secretion has been discussed. In 1978, cholinergic blockade by atropine was known to decrease insulin secretion mediated by gastric inhibitory polypeptides and gastrin release[77,78]. The action of atropine on gastric inhibitory polypeptides lowered postprandial insulin secretion. From the study published by Schafer *et al*[79] and Afonso *et al*[80], atropine inhibited the release of hepatic insulin-sensitizing substances, which therefore lessened insulin sensitivity during feeding. The parasympathetic nerves directly stimulate postprandial insulin secretion; therefore, atropine might play an inhibitory role in blood sugar control. However, a study by Svensson *et al*[81] showed that atropine improved insulin sensitivity in both lean and obese subjects. In the atropine-treated group, glucose uptake was higher than that in the subjects treated with saline alone. In summary, parasympathetic blockade might directly decrease insulin secretion mediated by gastric inhibitory polypeptides and delay intestinal emptying under cellular dehydration conditions[82]. However, atropine might improve insulin sensitivity based on the clinical trial mediated by Svensson *et al*[81]. Since atropine might only be given in the acute intoxication of organophosphate conditions, the acute adverse effect might not be potentiated.

ROLE OF ROS GENERATION IN ORGANOPHOSPHATE-MEDIATED NEW-ONSET DIABETES MELLITUS

From the evidence mentioned above, the oxidative stress generated by organophosphate increased gluconeogenesis and decreased insulin sensitivity. Therefore, interventions to lessen ROS generation have been proposed to prevent the development of organophosphate-mediated diabetes mellitus. N-Acetylcysteine is a widely used scavenger for ROS due to its regeneration of glutathione. From clinical trials, N-acetylcysteine has been applied to treat acute organophosphate intoxication.

From the clinical trial reported by El-Ebiary *et al*[83], n-acetylcysteine could achieve less atropine use and shorter hospitalization stays in acute organophosphate intoxication subjects. Falach-Malik *et al*[84] demonstrated that in diabetes-prone mice treated with a high-fat diet, n-acetylcysteine alleviated glucose intolerance by lessening hepatic steatosis. Charron *et al*[85] also demonstrated that in high-fat diet-fed maternal mice, n-acetylcysteine supplementation in the maternal stage decreased diabetes mellitus development in offspring. A similar effect was also demonstrated in a type 1 diabetes mellitus animal model under insulin deficiency[86]. N-Acetylcysteine also lessened organophosphate-mediated toxicity *in vivo*. A report from Yurumez *et al* [87] demonstrated that N-acetylcysteine could rescue antioxidative glutathione, nitrite and nitrate and decrease MDA generation in organophosphate-treated mice. The study conducted by Bayir *et al*[88] demonstrated that in organophosphate-poisoned mice, n-acetylcysteine alone could restore the cholinesterase concentration within erythrocytes, and the liver MDA level was lessened in n-acetylcysteine-treated mice rather than pralidoxime-atropine-treated mice or sham mice. From the aspect of decreasing organophosphate-mediated oxidative stress and the sequential development of diabetes mellitus, a therapeutic strategy for lowering ROS should be considered.

FUTURE PERSPECTIVES ON THE PREVENTION OF ORGANO-PHOSPHATE-MEDIATED NEW-ONSET DIABETES MELLITUS: RISK FACTOR STRATIFICATION

Since the development of diabetes mellitus is common in organophosphate-exposed subjects, risk stratification should be emphasized. The specific brand of organophosphate pesticide could influence the development of diabetes mellitus. Juntarawijit and Juntarawijit[89] noticed that endosulfan, mevinphos, carbamate and one fungicide (benlates) contributed to the development of diabetes mellitus in the Thai population. Apart from the specific insecticides, the environmental heavy metal content might play a synergistic role in the development of diabetes mellitus. From the study by Velmurugan *et al*[90], arsenics could synergize with organophosphate-mediated diabetes mellitus. At the same time, genetic polymorphisms should play a role in the development of organophosphate-induced diabetes mellitus. As the previous section mentioned, organophosphates could be metabolized by hepatic cytochrome p450, and metabolites might generate genotoxicity if the polymorphism existed within the subjects[91]. The first pass effect of cytochrome p450 generates toxic oxon organophosphate, which would be further oxidatively cleaved by cytochrome or hydroxylated by paraoxonase-1[92,93]. From the study by Al-Hakeem *et al*[94], the polymorphism in paraoxonase-1 with glutamine 192 to arginine made the subject vulnerable to gestational diabetes mellitus. The evidence shows a link between the polymorphism and organophosphate-mediated diabetes mellitus. In addition to diabetes mellitus development, lipid metabolism might be altered by paraoxonase-1 polymorphisms. The study conducted by Onat and *et al*[95] Leonel Javeres *et al*[96] demonstrated that the paraoxonase-1 polymorphism with the rs662 genotype was associated with ApoA1 and ApoB, which also reflected dyslipidemia in metabolic syndrome. Finally, personal protective equipment plays an important role in moderating the organophosphate metabolites associated with insulin resistance. Seesen conducted a study analyzing urinary organophosphate metabolites in pesticide sprayers and nonfarm workers[97]. In this study, the pesticide sprayer had a higher incidence of insulin resistance, and the only different organophosphate metabolite was diethylthiophosphate. No correlation was identified between diethylthiophosphate and the severity of insulin resistance. However, personal protection equipment lowered organophosphate metabolite generation. Personal protective equipment might play a preventive role in alleviating insulin resistance in organophosphate intoxication subjects.

CONCLUSION

Organophosphate pesticides have been linked to both acute and chronic intoxication. In acute intoxication, organophosphate-mediated cholinergic crisis might sequentially be followed by intermediate syndrome. Intermediate syndrome might hamper chronic muscle wasting and sarcopenia, therefore increasing the risk of diabetes mellitus. With chronic exposure to organophosphates, diabetes mellitus might develop by direct

damage to the pancreas and insulin resistance mediated by lipolysis, oxidative stress and chronic inflammation. Distal organ damage, such as acute kidney injury, might worsen possible organophosphate-mediated diabetes mellitus. The standard therapeutic strategy for cholinergic crisis may play a controversial role in managing organophosphate-mediated diabetes mellitus. However, reducing ROS might be a possible therapeutic strategy. In addition, elucidating the possible genetic polymorphisms to predict the development of diabetes mellitus with organophosphate intoxication might be essential.

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Anti-diabetics and antimicrobials: Harmony of mutual interplay

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Abstract

Diabetes is one of the four major non-communicable diseases, and appointed by the world health organization as the seventh leading cause of death worldwide. The scientists have turned over every rock in the corners of medical sciences in order to come up with better understanding and hence more effective treatments of diabetes. The continuous research on the subject has elucidated the role of immune disorders and inflammation as definitive factors in the trajectory of diabetes, assuring that blood glucose adjustments would result in a relief in the systemic stress leading to minimizing inflammation. On a parallel basis, microbial infections usually take advantage of immunity disorders and propagate creating a pro-inflammatory environment, all of which can be reversed by antimicrobial treatment. Standing at the crossroads between diabetes, immunity and infection, we aim in this review at projecting the interplay between immunity and diabetes, shedding the light on the overlapping playgrounds for the activity of some antimicrobial and anti-diabetic agents. Furthermore, we focused on the anti-diabetic drugs that can confer antimicrobial or anti-virulence activities.

Key Words: Diabetes; Immune disorders; Anti-diabetics; Antimicrobials; Anti-virulence

Core Tip: Understanding the mutual interplay between diabetes and microbial infection is necessary to control both and to avoid a lot of serious complications that may happen in such clinical conditions. Repurposing of approved drugs and investigation of their new application represents a promising approach for maximizing treatment outcomes.

In this review, we shed light on the overlapping areas of efficacy between anti-diabetics and antimicrobials.

INTRODUCTION

Diabetes is a chronic metabolic disorder associated with high blood glucose levels. Diabetes is a lifelong condition which requires proper monitoring and change in the patient's diet and routine habits. The world health organization reported an increase in the incidence of diabetes over the last decades worldwide that affected all social, economic and ethnic backgrounds. The universal prevalence of diabetes has nearly doubled since 1980 to 2014, rising from 4.7% to 8.5% in the adult population, and expected to rise to 10.4% in 2040[1]. This alarming uprise in the statistics of diabetes is owed to the global shift towards urban habits generally characterized by unbalanced diet, stress and reduced physical activity. Before the coronavirus disease 2019 (COVID-19) pandemic 650 million adults (13% of the world's adult population) were obese and it was estimated that 19.7% of the world's population will be obese by the year of 2030 [2]. The COVID-19 pandemic has been associated with increased risk of obesity and associated health hazards mainly diabetes. The global application of quarantine requirements forced billions of people into a new life style of isolation where people are forced to spend more time indoors with minimum physical activities and limited contact with others. The quarantine related frustration pushed people to consume larger amounts of high sugar foods which is reflected as higher incidence of obesity[3, 4]. Moreover, many studies have outlined the role of obesity and diabetes as important risk factors in COVID-19 infections[5,6].

Diabetes is commonly divided into two major categories depending on the age of onset and the pathophysiological cascade of events giving rise to diabetes; type I diabetes (T1DM), also known as juvenile diabetes, is characterized by the inability of pancreas to secrete insulin due to damage of β-cells mostly caused by an autoimmune disorder. The onset of T1DM appears usually in childhood and requires lifelong insulin injections. On the other hand, type II diabetes (T2DM) is characterized by insulin resistance that can be accompanied by reduced insulin secretion from the pancreas. T2DM is more common than T1DM, its onset appears in adulthood and its treatment involves diet control, medications for control of blood glucose level and eventually insulin injection is required in late stages[7].

The delay in diagnosis and treatment of diabetes can lead to irreversible damage to many of the body organs; some of the complications of diabetes include neuropathy, retinopathy, nephropathy, cardiovascular diseases, peripheral insufficiency, and diabetic foot ulcers. Failure to control diabetes can eventually lead to life threatening complications like kidney failure, lower limbs gangrene, heart attacks and stroke[8]. Some of the most distinguished complications of diabetes include: Immunodeficiency, high risk of infection and longer recovery period, all of which represent lifelong companions of diabetic patients. The most frequent infections in diabetic patients are respiratory tract infections, urinary tract infections, skin and soft tissues infections, diabetic foot ulcers, otitis and periodontal infections[9]. Many mechanisms were proposed for the reasons behind the high risk of contracting infection in diabetic individuals like the high blood glucose level, the lower-than-normal pH in body fluids [9,10], in addition to poor vascularity of peripheral tissues[11], all of which support pathogenic infestation. However, the most profound factor is the impaired immune functions. The relation between microbes and diabetes is bidirectional. In other words, the high blood glucose levels complicate infections and also some microbial infections can contribute to the etiology of diabetes[12-16]. The undeniable role of inflammation in both diabetes and infection have been extensively portrayed, which lead to the conclusion that the use of anti-inflammatory agents can represent a rationale treatment approach for better control of diabetes or even delaying its complications. Generally, anti-inflammatory agents are essential members in any anti-diabetic regimen; non-

steroidal anti-inflammatory drugs and salicylates are commonly prescribed anti-inflammatory agents for better control of the diabetes associated inflammation[17].

As diabetics are immunocompromised chronic patients and are more susceptible to microbial infections, exploring antimicrobial activities of approved anti-diabetic agents may be highly appreciated by clinicians. Preferential selection of anti-diabetic agents that have additional antimicrobial activities for diabetic patients can offer multiple advantages, including antimicrobial protection enhancement and decreasing the treatment costs[18-21]. In this work we intend to discuss the multiple facets of the relation between infection and diabetes. We shed the light on the interplay between immunity, diabetes and microbial infections, discussing the influence of diabetes on worsening of microbial infections. Additionally, the antimicrobial agents that can harbor anti-diabetic activities were discussed, with special interest in the anti-diabetic drugs which have antimicrobial activities, enhance immune responses or mitigate microbial virulence (Figure 1).

DIABETES AND IMMUNITY INTERPLAY

The interplay between immune-dysfunction and diabetes has a deep complicated background that exceeds our full understanding. The question whether immune-dysfunction is the cause or the effect of diabetes was always questioned. It is widely accepted that an immune disorder is responsible for T1DM *via* T cell-mediated selective destruction of pancreatic β-cells[15]. The activation of such destructive autoimmune behavior is based on a genetic factor in addition to a triggering environmental event[22]. Meta-analysis of genomic data has identified the genetic loci related to high risk of T1DM, the most prevailing are haplotypes in human leukocyte antigen class II, other common risk loci include mutations in *INS*-gene leading to preproinsulin misfolding and polymorphisms in Protein tyrosine phosphatase, non-receptor type (PTPN-22), interleukin-2 (IL-2), renalase (RNLS) and *CTLA-4* genes[9,15,23]. The presence of a single or multiple risk loci would lead to an unfortunate sequence of immunological reactions starting by loss of tolerance to pancreatic islets β-cell antigens, the production of anti-diabetic islet antibodies from plasma B-cells and the active involvement of autoreactive CD4- and CD8-T cells, eventually leading to steady rate damage in pancreatic β-cells[24]. This steady autoimmune destructive pattern remains hidden from the individual up until a critical damage limit is reached in the pancreatic islets after which hyperglycemia prevails and external insulin dependence becomes crucial[25]. During the prolonged silent preclinical period, early detection and reversal of the disease is possible by screening for the genetic risk loci, circulating anti-islet autoantibodies, and auto-reactive CD4 and CD8-T cells[15,25,26]. The presence of high-risk genetic loci only presents a predisposing factor to the disease; as a matter of fact, an individual carrying a high-risk gene could enjoy a delay in the onset of symptoms if he was lucky enough to escape the triggering factors associated with T1DM[22,27]. The triggering event can be an alteration in gut microbiota, obesity, a dietary factor like gluten or early introduction of cow's milk in infancy, toxins or a viral infection especially by dsRNA virus[28-31]. It is widely conceived that such events could trigger abnormal immunogenicity of β-cells and the loss of tolerance to pancreatic islets β-cell antigens which marks the onset of the autoimmune response[22,24,27].

On the other hand, T2DM etiology involves weaker dependence on genetic factors and more correlation to life style factors[32]. The genetic risk factors predisposing to T2DM were outlined by genome-wide association studies as polymorphisms in *TCFL2*, *ABCC8*, *CAPN10*, *PPAR*, *CDNKN2A/B*, *CDKAL1*, and *IGF2BP2* genes[33]. How- ever, the genetic susceptibility factor plays little role in the development of T2DM and again immunity related disorders play the leading role in the pathogenesis of the disease[9]. T2DM is generally characterized by a chronic low grade of inflammation arising from the immune response to hyperglycemia, aging, obesity and stress[7,34]. A growing mass of evidence suggests the involvement of both innate and adaptive immune-responses in the inflammatory trajectory of T2DM. The diabetes related dysfunctions in adaptive immunity include decreased γδ-T cell function, increased inflammatory T-helper phenotypes, decreased regulatory T-cells, and impaired B-cells function[7,35-37]. On the other hand, T2DM innate response defects come with altered neutrophil function, increased pro-inflammatory M1 macrophages, abnormal natural killer cell phenotypes, and increased inflammatory dendritic cells[7,35,36,38]. It should be noted that systemic inflammation is less projected in T1DM due to stimulated production of IL-10 from dendritic cells which is reflected as low incidence of insulin resistance[7,
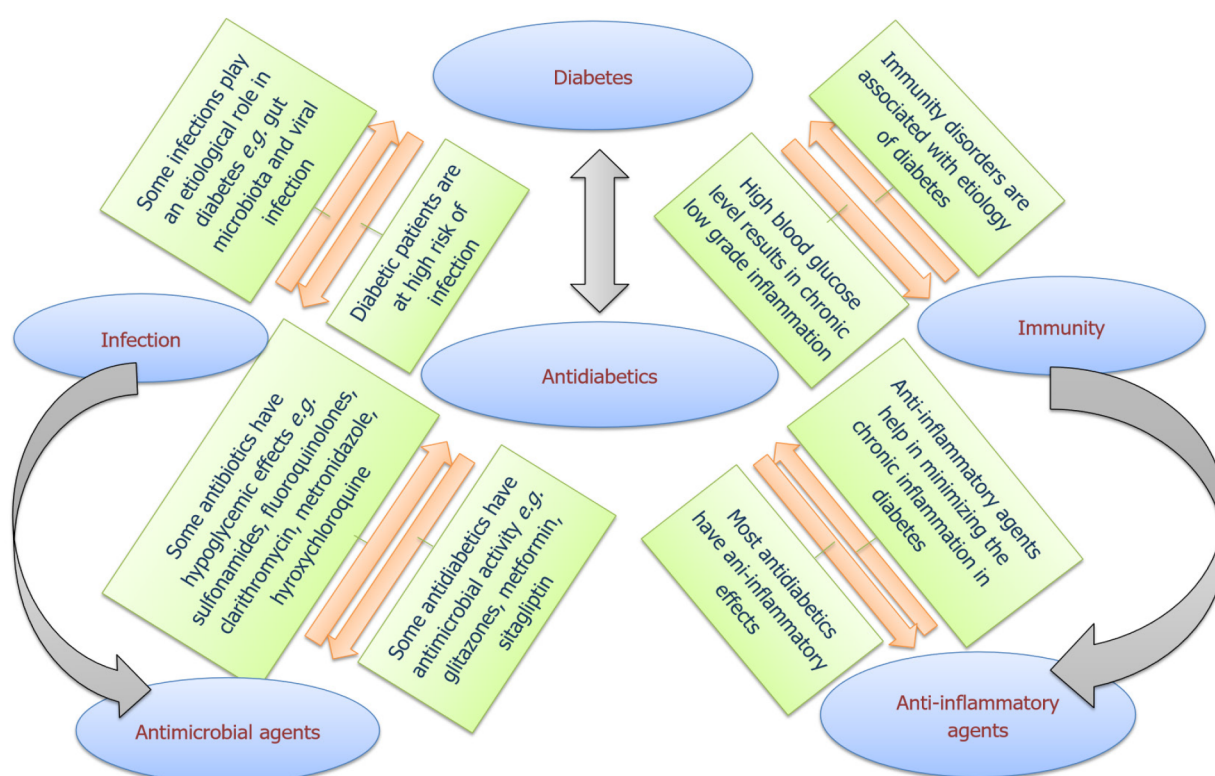
Figure 1 Interplay between diabetes, infection and immunity.

36]. Moreover, an autoimmune response characterized by circulating autoantibodies has been increasingly recognized in many T2DM patients[35,36,38]. However, the autoimmune pathway differs in T1DM compared to T2DM, since autoimmunity in T2DM patients is more related to obesity-activated chronic inflammatory responses and β-cells fatigue[36,38].

MICROBIAL INFLUENCE ON DIABETES

Imbalance in gut microbiota and diabetes

The gut microbiota represents an ecosystem of trillions of inhabitants that co-exist in our gastrointestinal track in perfect balance with other body systems, actively engaging in a mutually beneficial relationship[39,40]. The formation of the gut microbiota starts in infancy and continues to develop and diversify throughout our lifetime[41]. The complex and dynamic population of the gut microbiota includes bacteria, fungi, protists, archaea, and viruses with bacteria comprising the vast majority in the gut population[42]. The composition of the gut microbiota is subjected to continuous alterations and development depending on age, diet, geographical distribution, infection history, antimicrobial treatments, medication regimen, stress and physical activity among many other parameters[43,44], leading to huge composition variability between individuals in a pattern that can resemble fingerprint uniqueness[45]. Indeed, the gut microbiota plays an undeniable role in many metabolic and immune related disorders *e.g.*, metabolic syndrome, diabetes, inflammatory bowel diseases and obesity[46-48]. However, the exact contribution of gut microbiota to the pathophysiology of diabetes is widely variable due to the individual variations on the matter. That being said, a handful of gut microbiota members have shown repeated signals in multiple researches, where the results suggested some bacterial genus to impact protective effects against T2DM *e.g. Bifidobacterium, Lactobacillus*, *Bacteroides, Roseburia*, *Faecalibacterium*, *Clostridium* cluster IV and subcluster XIVa and *Akkermansia*[49], such members were suggested as probiotics treatment with high association to improved glucose homeostasis and protection against T2DM, bearing in mind the importance of species-dependent variation in the result outcomes[49,50].

The suggested mechanisms for the protective effect of these bacterial groups against T2DM involves multiple pathways; observations have recorded an increase in the anti-

inflammatory cytokines IL-10 and IL-22, enhanced T regulatory cell function, increased transforming growth factor-beta (TGF-β), suppressed intestinal inflammation, decreased gut permeability and increased insulin sensitivity[49]. On the other hand, other bacterial genera were repeatedly associated with impaired glucose homeostasis and increased risk of obesity and diabetes *e.g.*, *Ruminococcus*, *Fusobacterium*, *Blautia* and *Firmicutes*[49,51,52]. The involved mechanisms are not clearly elucidated; however, some studies suggested that the introduction of a dietary factor like gluten, unbalanced high fat diet or the reduction in gut pH can significantly increase the pro-diabetic bacterial population at expense of the balance and diversity in the gut microbiota[14], with many observations relating these effects to increased pro-inflammatory cytokines and induction of antigen-specific T cells-initiated destruction of the pancreatic β-cells in T1DM[14,16], increased bowel permeability[53], endotoxemia[54,55], and altered metabolism of bile acids[56,57]. Moreover, the shift in balance in the gut microbiota can lead to overgrowth of bacteria that has an increased capacity to harvest energy from the diet, such members of microbiota can boost energy uptake from diet by hydrolysing the undigested plant polysaccharides (cellulose, xylan and pectin) thus contributing to higher risk of obesity and subsequently higher risk of T2DM[58,59].

MICROBIAL INFECTIONS TRIGGER DIABETES

Many of the risk factors related to diabetes have been studied and identified like the genetic risk loci, obesity and stress among others. Nevertheless, the role of some microbial related events has been repeatedly outlined as potential triggers in both T1DM and T2DM. In the following segment we discuss examples of the identified microbial suspects in the etiology of diabetes.

One of the leading triggers of T1DM is believed to be an enterovirus infection by Coxsackievirus B (CVB), rotavirus, mumps or cytomegalovirus[60-62]. This idea was first conceived when an observation in the Finnish population, where the highest incidence of T1DM is reported, lead to linking the first signs of autoantibodies in genetically susceptible children to the seasonal pattern of enterovirus infections, especially by CVB-1[13,60]. Additionally, enteric infections by CVB-4 were repeatedly associated with pancreatic islets inflammation and infiltration mediated by β-cell specific autoantigens and subsequent β-cell apoptosis[62]. Another study has outlined the positive correlation between enterovirus (A) overpopulation in the gut and an autoimmune response in the pancreatic islets of genetically susceptible individuals [63]. Some enterovirus can directly infect the β-cells *via* targeting specific pancreatic receptors such as the poliovirus receptor and integrin αvβ3, hence initiating an inflammatory autoimmune response[64,65]. This effect was clearer in some individuals who suffer a chronic viral induced β-cell inflammation that can be detected by tracing enteroviral major capsid protein VP1, enteroviral RNA and the over-expression of the major histocompatibility complex-1[12,66,67].

The association between hepatitis C virus (HCV) and T2DM was repeatedly studied; it is known that some extra-hepatic manifestations of HCV are related to impaired glucose homeostasis, decreased glucose uptake and increased insulin receptor damage[68,69]. Molecular investigations into the underlying mechanisms have revealed that HCV core protein enhances the production of reactive oxygen species (ROS) in the mitochondria and endoplasmic reticulum of hepatocytes. The accumulating oxidative stress results in propagating hepatic cirrhosis and fibrosis with impairment in liver mediated glucose homeostasis[70]. HCV core protein also activates serine phosphorylation with subsequent deterioration of insulin receptor substrate (IRS)-1 and consecutive blocking of insulin signal propagation at the insulin receptors [68,71]. Additionally, the function of (IRS)-1 is further impaired due to degradation mediated by the inflammatory mediator tumour necrosis factor (TNF)-α[69,71]. Moreover, HCV induces gluconeogenesis, reduced glucose uptake and accumulation of lipid droplets *via* up-regulation of the enzymes glucose 6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase 2 (PCK2), and down regulation of glucose transporters (GLUT)-2 and (GLUT)-4[68]. The preceding information leads to the general conclusion that treatment of HCV infection could impose improvement in glucose homeostasis and insulin resistance, that was indeed observed in patients receiving anti-HCV antiviral regimens as shall be discussed shortly.

In an epidemiological study, an inverse relationship has been established between the decreasing prevalence of helminth infections and the increasing prevalence of metabolic diseases as diabetes[9]. But the controversy about the influence of *Helico-*

bacter pylori (*H. pylori*) bacteria on diabetes is more interesting. *H. pylori*, Gram-negative bacteria, is the most common causative agent of peptic ulcer and chronic gastritis[72]. *H. pylori* infection persists in the gastric epithelium generating local and systemic inflammation induced by multiple mediators[73-76] in addition to molecular antigenicity which provokes autoimmune responses[77,78]. All of these abnormalities predispose to a storm of inflammatory manifestations that has been linked to multiple extra-gastrointestinal disorders such as diabetes, cardiovascular disease, metabolic syndrome, atherosclerosis, neurodegenerative disorders, idiopathic iron deficiency anemia and vitamin B12 deficiency[79]. The relation between *H. pylori* infection and diabetes was proposed and discussed multiple times with conflicting significances being presented. Multiple meta-analyses have established positive correlation between chronic *H. pylori* infections and T2DM with less significant correlation to T1DM[80,81]. The correlation was more obvious in studies performed on data from Asian, European and African cases, with contradictory results obtained from United States patients[80, 81]. There are two main proposed mechanisms for the diabetogenic effect of *H. pylori*: The diffuse inflammation stress induced by the infection and gastric hormones imbalance[82,83]. It can be expected that the pro-inflammatory environment caused by *H. pylori* would impact insulin receptors, leading to impaired insulin sensitivity. This hypothesis was confirmed in multiple studies that highlighted the positive correlation between *H. pylori* infections and insulin resistance[84,85]. Furthermore, one study reported that the presence of *H. pylori* antibodies was linked to 2.5 higher levels of insulin resistance[86]. *H. pylori* infection was also associated with higher incidence of chronic complications in T2DM patients, and associated with higher mean glycated hemoglobin (HbA1c), an indicator of chronic hyperglycemia in Prediabetic individuals [75]. On the other hand, it was reported that eradication of *H. pylori* by antibiotic treatment courses was not associated with improved insulin sensitivity[87]. In addition to the inflammatory pathways connecting *H. pylori* to T2DM, a parallel hormonal mechanism was proposed; *H. pylori* infection was reportedly associated with imbalance in secretion of the gastric hormones, with increased secretion of gastrin and decreased secretion of ghrelin, leptin and somatostatin[83,88,89]. The *H. pylori* induced imbalance in these hormones was associated with impaired insulin release from pancreatic islets, increased appetite and fat deposition[90-92]. More studies are required to clarify the exact pathways for *H. pylori* triggered insulin resistance and the interactions between gastric hormone imbalance and insulin release from the pancreatic islets, by which *H. pylori* interferes with insulin release from the pancreatic islets.

DIABETES PROMOTES MICROBIAL INFECTIONS

The evidence of bidirectional link between diabetes and viral, bacterial, fungal, and parasitic infectious agents has been proven and extensively documented[9,10,93,94]. This bidirectional link between diabetes and infection is governed by the inflammatory mediators that link inflammation process and diabetes vulnerability to infection[9,10, 23]. In other words, diabetes augments the outcome of microbial infections and vice versa (Figure 2). As a consequence of diabetes, immune alterations would lead to (1) Increased activity of ROS; (2) Increased production of the pro-inflammatory mediators TNF-α, INF-γ, IL-1β, IL-6, IL-8, IL-12 and IL-17; (3) Reduced protective effect of the anti-inflammatory mediators interferon-1, IL-2, IL-10 and IL-22; (4) Reduced expression of cathelicidins in macrophage leading to impaired bactericidal activity and chemotaxis; and (5) Reduced glutathione and non-enzymatic glycation of complement factor thus inhibiting its activation[7,9,35,36,38]. Such ramifications are responsible for impaired function of the first line antimicrobial defense, higher susceptibility to pathogens and delayed healing[7,35]. Long term hyperglycemia will cause advanced glycation end (AGE) products of proteins such as AGE-albumin which hinders trans-endothelial migration in macrophages[35].

Just as diabetes weakens both humoral and cellular immune responses, hyperglycemia can enhance the microbial virulence. Generally, diabetic patients with higher HbA_{1c} (> 6.5%) are at higher risk of hospital-acquired and community-acquired infections and sepsis[10,95,96]. Elevated HbA_{1c} represent a risk factor for bacteremia and sepsis in diabetic patients who suffer from urinary tract infections[96]. The increased susceptibility to *E. coli* infections is owed to glycation of *E. coli* fimbrial FimH adhesin which promotes the bacterial adhesion to urinary tract epithelial cells[97]. In periodontitis, diabetes enhances expression of IL-17 and increases pathogenicity of the oral microbiome[98]. In respiratory infections, the elevation of blood glucose in
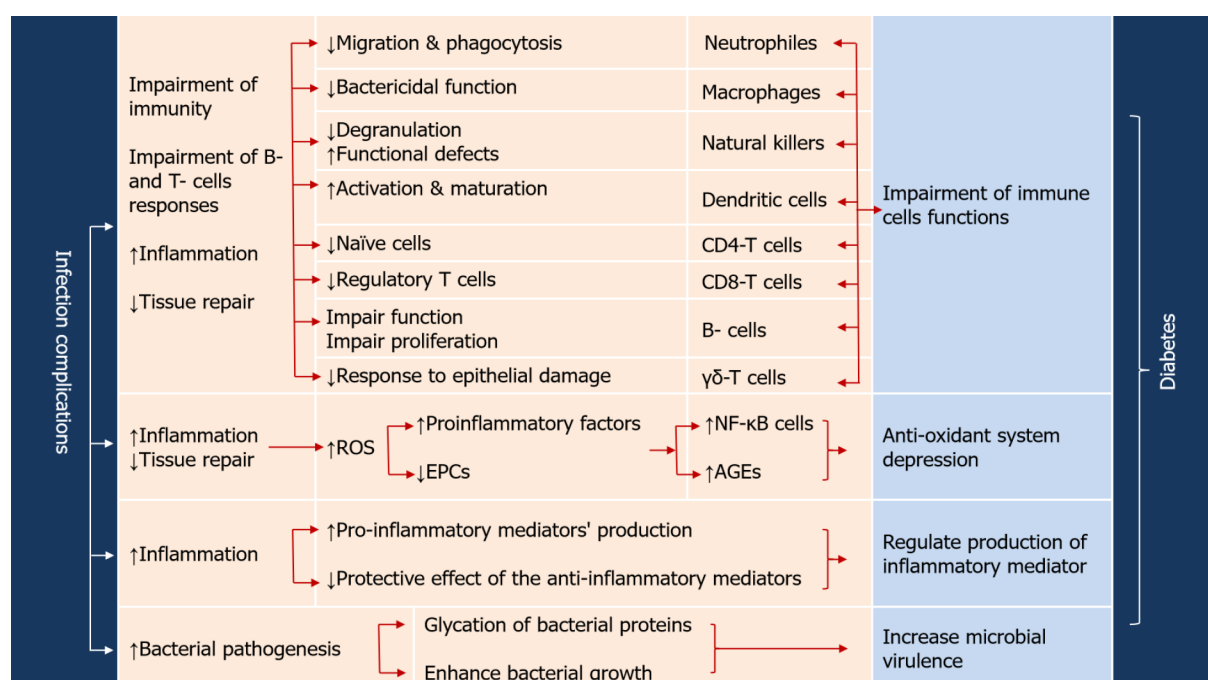
Figure 2 Some proposed mechanisms for the effect of diabetes on enhancement of microbial infections. NF-κB: Nuclear factor kappa B; AGEs: Advanced glycation end; EPCs: Endothelial progenitor cells; ROS: Reactive oxygen species.

diabetic mice promotes the *Staphylococcus aureus* growth in the airways increasing the possibility of infection[99]. Moreover, the influences of diabetes on patient's immunity are considered in defensing mechanisms against mycotic, parasitic and viral infections. For instance, *Candida* spp. constitute the most frequent isolates from urogenital tract of hyperglycemic patients[100,101] and the severity of infection is in correlation with glucose level[102]. The adhesion of *Candida* spp is enhanced in presence of high glucose which increases the expression of intercellular adhesion molecule-1[103]. Diabetes changes the morphology of *Leishmania major* lesions[104] and particularly causes severe cutaneous *Leishmania infantum* lesions[105]. Chickenpox complications such as postherpetic neuralgia are more severe and persistent in diabetics[106], and T1DM vascular complications confers an additional virulence to herpes zoster[107]. The severity of liver damage is more observed in HCV patients with uncontrolled glucose levels[108].

In context with prominent effects of diabetes on both innate and adaptive immunity, diabetic patients are more susceptible than nondiabetics to all types of infections such as nosocomial infections[95], sepsis[10] tuberculosis[109-111], *Legionella* infections [112], gum infections[113], fungal infections[114], dengue fever[115], influenza virus [116], herpes zoster[106,117,118], and other infections reviewed in[9]. Moreover, the risk of post-sepsis infections increases due to alterations in innate and adaptive immune responses resulting in chronic inflammation and persistence of causative microbe[10,96]. As a consequence of impaired immunity, diabetic patients are prone to more aggressive, recurrent and life uncommon life-threatening infections[9,10,95,119, 120]. Conclusively, diabetes augments the bacterial virulence either by impairing the patient's immune responses or even by enhancing the invasion and spreading of bacterial[9,10,120,121].

Diabetes complications favor the microbial pathogenesis due to decreased blood supply to the affected areas and reduced neural sensation[122]. Furthermore, the development of resistance to antimicrobial agents is more common in diabetic patients as compared to nondiabetics[123]. The prevalence methicillin-resistant *S. aureus*[124-126], vancomycin-resistant *Enterococci*, carbapenem-resistant *Enterobacteria*, extended-spectrum β-lactamases-producing *Enterobacteria*, and non-fermenting Gram-negative bacilli are elevated in diabetic patients[9,120,127,128]. This is owed to the impaired immunity of diabetic patients which principally leads to increasing their susceptibility to infectious agents and failure in complete eradication of persistent infections, this results in more exposure to antimicrobial agents and subsequently higher risk of antimicrobial resistance. Examples for the antimicrobial resistance development in treatment of surgical infections and diabetic foot are tremendous and very serious as reviewed[9,10,120,129]. Although *H. pylori* is well known for its susceptibility to usual

therapy regimen, it resists eradication in diabetics which require specific modified antimicrobial regimen[130].

ANTIMICROBIALS WITH ANTI-DIABETIC ACTIVITY

Antimicrobial agents can induce multiple pharmacological effects beyond their lethality to invading pathogens; these effects can be reflected as metabolic changes which sometimes affect glucose homeostasis. Recently, it was shown that the exposure to antibiotics in childhood has been linked to increased risk of metabolic disorders later in life and associated with changes in development of pancreas[63]. Additionally, some antibiotics may alter the antidiabetic plasma levels; diabetic patients with tuberculosis were advised to take rifampicin and metformin with sufficient time interval[131]. Clarithromycin is another antimicrobial agent associated with severe hypoglycemia in diabetic patients receiving hypoglycemic medications, the risk increases with renal impairment and in elderly patients[132]. The suggested mechanism for clarithromycin induced hypoglycemia is the inhibition of the cytochrome-P450 enzyme which is responsible for metabolic inactivation of sulfonylurea or meglitinide hypoglycemics, this leads to increased plasma concentration of these medications and subsequent hypoglycemia[133]. Similar hypoglycemic effects were reported for metronidazole which also inhibits CYP2C9 inhibitor which interferes with the metabolism of hypoglycemic agents[134]. It can be concluded from the above that clarithromycin and metronidazole don't have a direct hypoglycemic effect, rather they increase the systemic concentration of sulfonylurea or meglitinide drugs as a result of the delay in their metabolism[134]. More considerably, some antibiotics impose disrupting effects on gut microbiota with alterations in the expression of their key metabolic pathways which influences both their response to antibiotics and the glucose metabolism[135-137]. In addition to the above mentioned indirect hypoglycemic effects of some antimicrobial agents, others have showed direct hypoglycemic effects. In the next paragraphs we will give a glance at some of these drugs.

Sulfonamides are of the oldest known antimicrobial agents. During their long use, clinical observations revealed other clinical effects of sulfonamides including anti-carbonic anhydrase, anti-obesity, diuretic, hypoglycemic, antithyroid, antitumor, anti-neuropathic and anti-inflammatory activities[138]. The hypoglycemic activity of some sulfonamides received the most attention from the mid-20th century scientists, it was concluded that sulfonylureas have the best hypoglycemic activity through stimulating insulin secretion from pancreatic β-cells and decrease in hepatic clearance of insulin [139]. Further investigations into the hypoglycemic effects of sulfonylureas lead to the development of first and second generations of hypoglycemic sulfonylureas which constitute an important group of anti-diabetic agents that are still widely used today for the treatment of T2DM[140]. It was repeatedly advised to be cautious while combining sulfonamides with other hypoglycemic agents due to the synergistic effects that can lead to life threatening hypoglycemia, which is more common in case of elderly and renal dysfunction patients[141-143].

Fluoroquinolones represent a group of broad-spectrum antimicrobial agents that are widely used for treatment of respiratory tract and urinary tract infections. Despite the fact that this group has outstanding antimicrobial efficiency against a wide range of infections, they suffer from serious risk factors like the significant risk of aortic aneurysm, neuropathy, tendinopathy, and interference with glucose homeostasis[127, 144,145]. Fluoroquinolones can induce life threatening hypoglycemia in diabetic patients, and dysglycemia in nondiabetic individuals. The suggested mechanism of hypoglycemia is *via* blocking the ATP-sensitive K+ channels in the pancreatic β-cell in the pancreas which boosts insulin secretion, however the mechanism behind the hyperglycemic effect is unclear[146]. The FDA repeatedly reported the high risk of dysglycemia associated with different members of fluoroquinolones. The multiple reports of severe hypo- and hyperglycemic clinical observations were the reasons behind the withdrawal of oral and systemic gatifloxacin preparations from the markets in 2006[147].

Hydroxychloroquine is an antimalarial drug that shows additional anti-inflammatory, immunomodulatory, anti-rheumatic and hypolipidemic activities. Hydroxychloroquine is also known to exert a significant hypoglycemic effect[148]. The exact mechanism of the hypoglycemic effect is not known; however, it is suspected to increase insulin receptors sensitivity, decrease hepatic clearance of insulin and reduce systemic inflammation[148-150]. Benzimidazoles are group of medications mostly

used as anti-helminthic. It was reported that they have hypoglycemic activity mediated by augmenting insulin secretion and activity[151,152].

Telaprevir is a protease inhibitor effective against HCV genotype 1. Some studies reported the development of hypoglycemia in diabetic patients receiving the antiviral telaprevir treatment course. One case study reported a female diabetic patient with obesity and HCV-related cirrhosis. She was given triple antiviral treatment by interferon-α, ribavirin and telaprevir. During the course of treatment multiple episodes of severe hypoglycaemia were recorded, however this effect disappeared after course completion which drove the general conclusion that telaprevir could impose a hypoglycemic effect[153]. Similar conclusions were obtained from another case study of a male diabetic patients receiving anti-HCV triple treatment with interferon, ribavirin and boceprevir. The study reported reversal of diabetes and termination of the anti-diabetic treatment after the successful viral treatment. The study suggests a relation between HCV and diabetes and possible reversibility of glucose abnormalities with successful eradication of HCV[154]. Two years later, similar outcomes were reproducible with another diabetic male also receiving triple HCV treatment in addition to anti-diabetic regimen including insulin and metformin. After the successful antiviral treatment, the patient was able to gradually withdraw insulin from his anti-diabetic treatment regimen and continued only the oral hypoglycemic linagliptin. This study weighs on the possibility of reduced glucose imbalance and even reversal of diabetes as an unexpected outcome after the antiviral treatment, the study attributes the improved glucose homeostasis due to retained normal functions of the liver after the termination of the viral infection[155]. Similar conclusions were reached in another retrospective study that included 65 diabetic patients subjected to the anti HCV triple-treatment including sofosbuvir, the results indicated improved blood glucose levels in all enrolled cases after the antiviral treatment as a result of retained normal liver activity[156].

ANTI-DIABETICS WITH ANTIMICROBIAL ACTIVITIES

Just as there are antimicrobials that can induce dysglycemia, some anti-diabetic can alter the antibacterial metabolism[157]. Searching into new fields for application of currently approved medicinal drugs, scientists have been more interested in drug repurposing as an elegant strategy for applying maximum use of already approved medicinal agents[158,159]. The benefits may be augmented by repurposing routinely used anti-diabetics as antimicrobial agents, this decreases the dose and number of administrated drugs that results in saving time and cost, decreasing the drug-drug interactions and enhancing the patients' compliance with the applied treatment regimens.

Likewise, most of the commonly used anti-diabetic agents offer additional anti-inflammatory activity as a favorable side effect during the treatment; the anti-inflammatory properties of glitazones, metformin, sulfonylureas and Dipeptidyl peptidase (DPP)-4 inhibitors were authenticated and appraised. The hypoglycemic effect of these agents is usually associated with decreased oxidative stress, decreased pro-inflammatory and increased anti-inflammatory mediators[160].In this context, it is highly valuable to clearly identify anti-diabetic agents that have additional antimicrobial activity, which is considered an interesting and promising area of active research[18, 19]. During this search, we are interested in projecting the antimicrobial activities of some anti-diabetics (Table 1).

INSULIN

Insulin is a peptide hormone produced by β-cells of the pancreatic islets. It regulates glucose metabolism in all body cells[161]. Yano *et al*[162], showed the antibacterial activity of insulin on surgical site *S. aureus* infection *via* restoring neutrophil phagocytosis and bactericidal activity[162]. In 1946, Bollenback and Fox[163] showed the antibacterial activity of protamine zinc insulin against *Lactobacillus arabinousus, S. aureus* and *E. coli*. they owed the antibacterial activity to the additive protamine sulphate not to insulin itself[163]. Similar conclusion was derived from another study performed on commercial U.S.P. insulin, the bactericidal activity against *Staphylococcus epidermidis*, *S. aureus* and *E. coli* was secondary to the preservatives placed in the insulin and not to the insulin itself[164]. In general, most studies suggest that insulin doesn't have a direct antimicrobial effect, rather an indirect antimicrobial effect can be

Table 1 Examples of antimicrobial activities of some antidiabetic drugs

Antidiabetic drug	Antimicrobial activity	Proposed mechanism	Ref.
Metformin	Antibacterial	Activation of the AMPK-mediated phagocytosis and production of mROS	[170]
		Disruption of the outer membrane permeability	[170]
		Down regulation of the Q.S encoding genes and mitigate the bacterial virulence	[19,186]
	Anti-TB	Increasing the production of β-defensin-2, -3 and -4 which diminish bacterial growth and multiplication	[178]
		Inhibition of mitochondrial complex-I which is analogous to mycobacterial NDH-I complex	[176]
		Activation of T regulatory and CD8 memory T cells responses activity	[177]
Sitagliptin	Antibacterial	Downregulation of the Q.S encoding genes, occupy the Q.S receptors and diminish bacterial virulence	[18,19,186]
	Anti-COVID-19	Reduction of the inflammation intensity	[190,191]
		Targeting viral proteins	[192]
		Binding to viral spikes	[193]
	Antibiofilm	Targeting enzyme XPDAP, analogous to mammalian enzyme DPP IV	[187]
Vildagliptin	Antibiofilm	Targeting enzyme XPDAP, analogous to DPP IV	[187]
	Anti-amoebic	-	[188]
Saxagliptin	Antibiofilm	Targeting enzyme XPDAP, analogous to DPP IV	[187]
Pioglitazone	Antibacterial	Increasing phagocytosis and production of reactive oxygen species in phagocytes	[197,198]
Tolbutamide	Antibacterial and antifungal	-	[205]
2nd generation sulfonylureas	Antifungal	Inhibition of the NLRP3 inflammasome	[206]
	Antibacterial	Prevention of inflammasome effector IL-1β	[207]
Glimepiride	Anti-amoebic	-	[188]
Repaglinide	Anti-amoebic	-	[188]
Glucagon-like Peptide-1	In HIV treatment	Reduction of HIV-associated metabolic adverse effects	[212]
α-glucosidase inhibitors	Antibacterial	Targeting bacterial glucosidase	[220]
	Antiviral and anti-COVID	Alter glycosylation in viral life cycle	[221]

mROS: Mitochondrial reactive oxygen species; IL: Interleukin; AMPK: Adenosine monophosphate-activated protein kinase; XPDAP: X-prolyl dipeptidyl peptidase; Q.S: Quorum sensing; COVID-19: Coronavirus disease 2019; HIV: Human immunodeficiency virus.

expected due to the adjustment of hyperglycemia and relief in inflammation and oxidative stress. It is noteworthy to highlight the intrinsic anti-inflammatory nature of insulin as opposed to the inflammatory downfalls of hyperglycemia, additionally insulin promotes protein and lipid biosynthesis thus improving wound healing. Moreover, insulin induces the expression of the anti-inflammatory cytokines IL-4/IL-13, IL-10 and down regulates the pro-inflammatory cytokines IL-6 and IL-10[17,165]. However, research groups are invited to further investigate the insulin effects on microbial growth and virulence.

BIGUANIDE (METFORMIN)

Metformin is a hypoglycemic drug used as first line treatment in T2DM. The hypoglycemic activity is owed to the suppression of hepatic glucose production, the reduced intestinal absorption of glucose and the increase in peripheral glucose uptake, however, the exact molecular mechanism of metformin is still the focus of active

research[166]. Metformin also showed multiple beneficial effects that extend beyond diabetes control, with increasing studies referring to anti-inflammatory, cardio- and nephro-protective, anti-proliferative, antifibrotic and antioxidant effects. Moreover, metformin was suggested as an anti-aging compound with promises of increased lifespan and delayed onset of aging-associated diseases[167-169]. The repurposing of metformin extended to explore its antimicrobial activity, which also presented promising antimicrobial effects. A late study has shown the ability of metformin to restore tetracycline susceptibility in multidrug resistant strains of *S. aureus*, *Enterococcus faecalis*, *E. coli*, and *Salmonella enteritidis* both *in vivo* and *in vitro*. The study proposed the disruption of outer membrane permeability in resistant bacteria as a mechanism for reversing the bacterial intrinsic resistance to tetracyclines. Furthermore, the study reported that metformin imposed anti-inflammatory and improved innate immunity responses due to activation of the adenosine monophosphate-activated protein kinase (AMPK)-mediated phagocytosis and production of mitochondrial ROS (mROS)[170]. Metformin is associated with reduced serum levels of C reactive protein (CRP) and monocyte release of TNF-α, IL-1β, IL-6, MCP-1, and IL-8 in pre-diabetic patients[171]. Another study reported elevated bactericidal and anti-inflammatory outcomes upon combining metformin with photodynamic therapy for the treatment of chronic resistant periodontitis[172].

The antibacterial activity of metformin also attracted the attention of scientists as an adjuvant in tuberculosis (TB) treatment regimens[173]. Meta-analysis studies revealed reduced mortality rates and improved treatment outcomes in diabetic patients subjected to anti-TB regimen combined with metformin as anti-diabetic drug, also metformin administration was linked to reduced risk of TB disease among diabetics [174,175]. One suggested explanation was based on the fact that metformin is an inhibitor of mitochondrial complex-I which is analogous to mycobacterial NDH-I complex, hence giving the way for another mechanism of bactericidal activity of metformin[176]. Another study suggested that metformin had an immunomodulating effect by activating T regulatory and CD8 memory T cells responses activity leading to decreased pro-inflammatory responses which is reflected as reduction in lungs' lesions [177]. In another study, metformin was observed to reduce TB bacilli load in lung epithelial cells in relation to increased production of β-defensin-2, -3 and -4 which restrain bacterial growth and multiplication[178]. Contrary to expected, metformin has an enrichment rather than inhibition effect on gut microbiota, shifting the balance towards more short chain fatty acids-producing bacteria which are reported to confer protection against inflammation, maintain intestinal barrier integrity and augment insulin production from b-cells due to stimulation of glucagon-like peptide 1 (GLP-1) secretion[179,180].

In recent studies, the anti-virulence effects of metformin have been extensively studied. Significantly, metformin mitigated the virulence of *Pseudomonas aeruginosa in-vitro*[19,181]. It reduced the production extracellular virulence enzymes such as protease, elastase and hemolysin and inhibited bacterial motility and biofilm formation. The anti-virulence activity of metformin was owed to its ability to downregulate the quorum sensing (Q.S) encoding genes[19]. Q.S is a process uses chemical language by which bacterial populations can communicate. This intercellular communication is performed through signaling molecules produced by bacterial cell called autoinducers that are detected by receptors on another cell. The Q.S signaling system controls various virulence factors and physiological functions in both Gram-positive and Gram-negative bacteria. Q.S targeting has been proposed as an effective strategy to cripple the bacterial virulence[18,182,183]. Despite the *in vitro* metformin success in mitigation of Q.S, it failed to confer the protection to mice from *P. aeruginosa*. The *in vivo* failure of metformin was owed to its chemical nature which changed by the change of pH during bacterial growth, these changes hinder the complete blocking of Q.S receptors by metformin molecule[19]. Taking in consideration that metformin was not tested in combination with other antibiotics and was used in sub-MIC concentrations (10 mg/mL) which can be increased to enhance its efficacy, we encourage research group for further investigation of anti-virulence effects of metformin.

DPP-IV INHIBITORS (GLIPTINS)

Gliptins are oral hypoglycemic medications used for management of T2DM, they act by selective inhibition of DPP-IV leading to increased plasma GLP-1 and gastrointestinal insulinotropic peptide (GIP) levels, hence increased β-cell activity and suppression of glucagon secretion[184]. The alteration effects of gliptins on the

composition of gut microbiota developed functional shifts in the microbiome, that improves the glucose homeostasis[185]. Interestingly, DPP-4 inhibitors are associated with reduced inflammatory effects in adipose tissue and pancreatic islets through reduced expression of inflammatory cytokines and adjusted macrophage activity[171]. Among the thirteen members of gliptins, sitagliptin has an attractive chemical structure that may antagonize the Q.S receptors, plus it is the most prescribed gliptin. Hegazy *et al*[186], investigated the sitagliptin effects on the virulence behavior of *Serratia marcescens*. Interestingly, sitagliptin showed a significant capability of quenching the bacterial virulence both *in vitro* and *in vivo via* significant downregulation of the virulence encoding genes[18,186]. These findings encouraged us to further investigate another gliptin member: Vildagliptin in comparison to sitagliptin on virulent *Pseudomonas aeruginosa*[18,181]. Despite the marked downregulation effect of both vildagliptin and sitagliptins on Q.S encoding genes, vildagliptin failed to attain significant inhibition of bacterial virulence *in vitro* and *in vivo* as compared to the effects of sitagliptin. Docking studies provided us with the satisfying explanation that sitagliptin structure offers better fitting onto Q.S receptors as compared to the weak association of vildagliptin on the same receptors. We hypothesized that the anti-virulence or anti-Q.S activity of sitagliptin is not only due to its down regulation of responsible genes, but also due to its ability to block the Q.S receptors[19].

The potent competitive inhibition of bacterial virulence determinants of other bacterial species by sitagliptin and other gliptins were demonstrated (unpublished data). In a separate work, saxagliptin, vildagliptin and sitgliptin decreased *ex vivo* the biofilm formation by dental caries causing odontopathogen *Streptococcus mutans*. As bacterial enzyme X-prolyl dipeptidyl peptidase (XPDAP) is analogous to mammalian enzyme DPP-IV, it was hypothesized that anti-protease activity of gliptins may affect XPDAP and bacterial growth[187]. In a separate prospect, vildagliptin reduced the numbers of viable *Acanthamoeba castellanii* that causes fatal granulomatous amoebic encephalitis. The amoebicidal activity of vildagliptin was significantly enhanced when formulated as vildagliptin-conjugated silver nanoparticles[188]. Surprisingly, some studies linked between reduction in COVID-19 mortality rates and treatment with sitagliptin[189]. Gliptins, especially sitagliptin, reduced the inflammation intensity and may control cytokine storms mostly *via* nuclear factor kappa B signaling pathway[190, 191]. Nevertheless, cheminformatics suggested sitagliptin as anti-severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)[192] as a result of the expected potential molecular binding between sitagliptin and viral spikes[193]. Nar *et al*[194], showed the ineffectiveness of gliptins against SARS-CoV-2 protease[194]. An enzymatic assay was performed to measure the sitagliptin, linagliptin, alogliptin and saxagliptin inhibitory effects on catalytic activity of SARS-CoV-2 main protease M^{pro}, significantly tested gliptins were inactive. They owed this inactivity due to lack of apparent structural similarity between M^{pro} and DPP-IV[194]. Regardless of the controversy about the efficacy of gliptins as anti-COVID-19, more investigations are required to explore whether gliptins harbor anti-viral activity or not. That being said, we consider gliptins in general and sitagliptin in particular to be promising targets for drug repurposing as bacterial anti-virulence agents.

GLITAZONES

Thiazolidinediones (TZDs), also known as glitazones, are a group of oral hypoglycemic agents used in T2DM. Glitazones work by restoring insulin sensitivity through the selective activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) which controls the transcription of genes regulating glucose and lipids metabolism[195]. Glitazones also exert anti-inflammatory effects through suppression of IL-6 and reduction in circulating CRP levels [196]. One study reported direct impact of TZDs *via* increasing phagocytosis by liver recruited macrophages, increased production of ROS in phagocytes and decreased serum pro-inflammatory cytokines (TNF-α, IL-12, IFN-γ)[197]. It was shown that pioglitazone has antibacterial activity against *Streptococcus pneumoniae*, *E. coli* and *Klebsiella pneumoniae*. Moreover, pretreatment of bacteria with a suboptimal concentration of pioglitazone enhanced the antibacterial activity of some antibiotics[198]. In another study, pioglitazone was used as an adjuvant to amphotericin B to ameliorate cryptococcosis in a murine model, that may indicate its promising application as an adjuvant for controlling fungal infection[199]. Interestingly, thiazolidinedione nucleus is present in several antimicrobial compounds, *e.g.*, antibacterial, anti-mycobacterium, anti-malarial and antiviral, which was reviewed[200]. However, the mechanism of the

antimicrobial activity is not fully understood since prokaryotes lack the PPAR-γ receptor which is the target site for glitazones, the antibacterial activity of glitazones may be owed to thiazolidinedione nucleus[197,200]. Although there are no deep studies on the antimicrobial or anti-virulence activities of glitazones, we predict that their antimicrobial activities are owed to thiazolidinedione nucleus.

SULFONYLUREAS AND MEGLITINIDES

Sulfonylureas antidiabetic drugs stimulate insulin secretion from the pancreatic β-cells, they bind to ATP-sensitive K-channels in the β-cell membrane, depolarizes the cells and open voltage-gated Ca^{2+} channels that results in insulin release. Sulfonylureas anti-diabetics, especially the second generation, are widely used in the management of T2DM[201]. Multiple antidiabetic sulfonylurea derivatives showed significant bactericidal[202,203] and fungicidal activities[203,204]. The first generation of sulfonylurea antidiabetic tolbutamide analogues were screened for their antibacterial and antifungal activities, they showed activity against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *C. albicans*[205]. Interestingly, Lowes *et al*[206], and others suggested repurposing the secondgeneration sulfonylurea anti-diabetics (glyburide, glisoxepide, gliquidone, and glimepiride) to treat fungal and bacterial infections[206-208]. They demonstrated the sulfonylurea anti-diabetics capability to inhibit activation of the NLRP3 inflammasome in various disease models such as vaginal candidiasis[206] and *Burkholderia pseudomallei* infection (melioidosis)[207]. Sulfonylureas were reported to decrease M1 macrophage activity and reduce IL-1β synthesis, pioglitazone is a direct PPAR-γ inhibitor that reduces adipose tissue inflammation[171]. It was supposed that sulfonylurea anti-diabetics prevent the release of major inflammasome effector IL-1β. Considerably, sulfonylurea anti-diabetics lack antimicrobial activity against *C. albicans* or *Burkholderia pseudomallei* and their anti-inflammatory activity was not specific to microbial infections, that means the possibility of repurposing these drugs against infectious and other immunopathological diseases[206,207]. Moreover, glimepiride was repurposed as amoebicidal agent, it decreased the numbers and encysts of *Acanthamoeba castellanii*[188].

Meglitinides mechanism of action closely resembles that of the sulfonylureas, they stimulate the insulin release from the pancreatic β-cells. Meglitinides are used orally and comprise nateglinide and repaglinide[209]. Unfortunately, there is a shortage in reports discussing the meglitinides' effects on both immune system and microbes. One study, the anti-amoebic activity of repaglinide was assessed against *Acanthamoeba castellanii*. It showed significant amoebicidal activity comparable to vildagliptin and glimepiride[188].

GLP-1 AGONISTS

Gut enteroendocrine cells release GLP-1 to control the meal related hyperglycemia through inhibition of glucagon and enhancement of insulin secretions. GLP-1 receptor agonists or incretin mimetics such as exenatide, liraglutide and albiglutide are used for the treatment of T2DM[210]. Liraglutide can lead to wight loss by changing the overall composition of gut microbiota as well as the relative abundance of weight-relevant phylotypes[211]. Generally, the hypoglycemic effects of GLP-1 agonists improve the immune status in diabetic patients to counteract the microbial infections. The associated metabolic activities of these drugs may be helpful in the treatment of human immunodeficiency virus (HIV)-associated metabolic adverse effects[212], and PEGylated exendin-4 has the potential to treat sepsis[213].

SODIUM-GLUCOSE COTRANSPORTER-2 INHIBITORS

Sodium-glucose cotransporter-2 (SGLT-2) inhibitors, represented by canagliflozin, empagliflozin and dapagliflozin, are hypoglycemic agents that work by increasing renal clearance of glucose through decreasing the renal tubular glucose reabsorption, hence reducing blood glucose level[214]. The interaction between SGLT-2 inhibitors and antibiotics is bidirectional, while the pharmacokinetic profile of SGLT-2 inhibitors may be influenced by co-administration of some antibiotics as rifampicin[215], they confer protection from gentamicin induced nephrotoxicity[216]. There are no reports

documenting direct anti-microbial activities of SGLT-2 inhibitors. On the other hand, SGLT-2 inhibitors associated glucosuria increases the risk of urogenital infections especially in postmenopausal women with T2DM[217].

α-GLUCOSIDASE INHIBITORS AND AMYLIN ANALOGS

α-glucosidase inhibitors (AGI) reversibly bind to oligosaccharide binding sites of glucosidase enzymes, resulting in delaying the polysaccharide degradation to glucose, slowing down the food digestion and decrease blood glucose levels after meals[218]. Amylin analogs such as pramlintide affect glucose levels *via* several mechanisms, including slowed gastric emptying, regulation of postprandial glucagon, and reduction of food intake[219]. It has been hypothesized that the interaction capabilities of AGI to glucosidase enzymes may be beneficial in targeting bacterial glucosidase [220] and altering glycosylation in viral life cycle, showing anti-viral activity against HIV, HBV and COVID-19[221]. However, we shortage in reporting antibacterial or antiviral activities of used antidiabetic AGI like acarbose, several studies indicated the antibacterial, anti-biofilm[222] and antifungal[223] activities of other similar AGI.

CONCLUSION

The relationship between diabetes, immunity and infection is complicated and bidirectional in most cases. This fact is clearly presented in the tangled interactions between diabetes and immunity disorders where each can potentially contribute to the other in a kind of "the egg or the chicken" dilemma. On a parallel basis, antimicrobial and anti-diabetic agents showed a grey area of overlapping activities that should be subjected to further investigations. It would be of great value to submit such information into practical applications by refining the currently used anti-diabetic regimens to include the anti-diabetic agents which offer antimicrobial protection as an accessory benefit. The systematic application of this approach would minimize the wide margins of morbidity and mortality usually associated with diabetes, in addition to reduced treatment costs and overall better treatment outcomes. In this review, we tried to aggregate the available information which would support this approach in addition to highlighting the proved antimicrobial activities of multiple anti-diabetic agents. By putting this information in the hands of clinicians and researchers, more attention can be paid during selection of the treatment options in order to offer diabetic patients the best outcomes and help in better containment of the emerging statistics of the diabetes pandemic.

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Effect of glycemic control on markers of subclinical atherosclerosis in patients with type 2 diabetes mellitus: A review

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Abstract

Cardiovascular disease is the predominant cause of death in type 2 diabetes mellitus (T2DM). Evidence suggests a strong association between duration and degree of hyperglycemia and vascular disease. However, large trials failed to show cardiovascular benefit after intensive glycemic control, especially in patients with longer diabetes duration. Atherosclerosis is a chronic and progressive disease, with a long asymptomatic phase. Subclinical atherosclerosis, which is impaired in T2DM, includes impaired vasodilation, increased coronary artery calcification (CAC), carotid intima media thickness, arterial stiffness, and reduced arterial elasticity. Each of these alterations is represented by a marker of subclinical atherosclerosis, offering a cost-effective alternative compared to classic cardiac imaging. Their additional use on top of traditional risk assessment strengthens the predictive risk for developing coronary artery disease (CAD). We, herein, review the existing literature on the effect of glycemic control on each of these markers separately. Effective glycemic control, especially in earlier stages of the disease, attenuates progression of structural markers like intima-media thickness and CAC. Functional markers are improved after use of newer anti-diabetic agents, such as incretin-based treatments or sodium-glucose co-transporter-2 inhibitors, especially in T2DM patients with shorter disease duration. Larger prospective trials are needed to enhance causal inferences of glycemic control on clinical endpoints of CAD.

Key Words: Glycemic control; Atherosclerosis; Type 2 diabetes mellitus; Cardiovascular disease; Carotid intima media thickness

Core Tip: Progression or even regression of atherosclerosis is possible in type 2 diabetes mellitus, especially at an early stage of the disease, with better glycemic control and use of newer agents, such as dipeptidyl peptidase 4 inhibitors and sodium-glucose co-transporter-2 inhibitors. Despite considerable evidence, especially for structural markers like intima media thickness or coronary artery calcification, and pulse wave velocity, larger and longer trials are needed to establish their clinical utility and correlation with clinical end-points.

INTRODUCTION

Cardiovascular disease (CVD) is the predominant cause of death in type 2 diabetes mellitus (T2DM). Generalized vascular disease is found even in asymptomatic patients with T2DM, who appear to have worse cardiovascular prognosis compared to healthy individuals. Increased cardiovascular risk cannot fully be attributed to the presence of traditional risk factors, such as dyslipidemia, hypertension, smoking or hyperglycemia, in these patients. Although existing evidence suggests the presence of a strong association between duration and degree of hyperglycemia and vascular disease[1], large trials have failed to show cardiovascular benefit of strict glycemic control in T2DM, especially those with longer disease duration[2,3].

Endothelial dysfunction is an early event in the progression of atherosclerosis[4]. Subclinical atherosclerosis, which is increased in T2DM patients, includes impaired vasodilation, increased coronary artery calcification (CAC), carotid intima media thickness (cIMT), arterial stiffness (AS) and reduced arterial elasticity. Each of these alterations is represented by a marker of subclinical atherosclerosis, serving as a cost-effective alternative to classic complex cardiac testing.

This is of great importance, given that the current diagnostic strategy is based on targeting traditional risk factors or using scoring systems that might either be insufficient to identify high-risk patients or present limited value in asymptomatic populations who lack these risk factors and yet suffer from CVD complications[5,6]. Therefore, imaging-guided risk assessment for detection of subclinical atherosclerosis might not only improve the compliance of those at high risk but also help reclassify lower risk patients who might benefit from targeted or more aggressive treatment. The Framingham risk score, an established tool for asymptomatic patients, appears to be less predictive for diabetics as opposed to the general population[7]. Therefore, increasing interest has led to the development of other screening tests for this population. As a subclinical marker of CVD, CAC scoring is known to predict cardiac events and has been a valuable tool for coronary artery disease (CAD) stratification of low–intermediate-risk patients, such as asymptomatic diabetics, and was recommended according to American Heart Association/American College of Cardiology Foundation guidelines[8].

In this review, we searched PubMed and Google Scholar for potentially relevant articles published from January 1, 1990 to December 31, 2020 with the following search terms: "subclinical atherosclerosis," "endothelial dysfunction in diabetes mellitus type 2," "effect of glycemic control in type 2 diabetes mellitus," "effect of glycemic control on subclinical atherosclerosis" and "HbA1c and markers of endothelial dysfunction."

STRUCTURAL MARKERS

cIMT-Carotid atherosclerosis

The use of B-mode ultrasound for cIMT assessment is a noninvasive, sensitive and

reproducible technique, which can be used to identify and quantify subclinical vascular disease and detect carotid plaques. It is considered a strong predictor for cardiovascular morbidity and mortality in T2DM and is used in numerous studies to detect patients at high risk of developing these complications.

Normally, cIMT increases with age, while male sex is associated with higher values [9]. The Atherosclerosis Risk In Communities (ARIC) study found a 0.07 mm increase of mean cIMT in all age groups of diabetic patients compared to nondiabetics after adjustment for other cardiovascular risk factors[10].

Interestingly, HbA1c is independently associated with cIMT values even in persons without diagnosed diabetes, suggesting the significance of glycemic control in development and progression of atherosclerosis[11]. It has become clear that, even in the prediabetic state, the risk of CVD is modestly increased[12,13]. In this direction, Di Pino *et al*[14] reported that, even in patients with prediabetes or newly onset diabetes, cIMT is impaired and correlates significantly with HbA1c. Moreover, higher HbA1c values are associated with higher cIMT values in subjects with normal glucose tolerance (NGT). Therefore, HbA1c is better than fasting glycemia or oral glucose tolerance tests as a surrogate marker to identify patients at high CVD risk.

In T2DM patients with near-normal HbA1c levels (5.8%–6.4%), further improvement of glycemic control prevents cIMT progression[15]. Recently, it has been suggested that poor glycemic control (*i.e.* HbA1c > 7%) and longer diabetes duration (> 1 year) independently exert adverse effects on cIMT in a smaller population (n = 45) of younger patients with diabetes (aged 10–25 years). The presence of hypertension and higher body mass index are predisposing factors as well[16].

Interestingly, Di Flaviani *et al*[17] investigated the effect of glucose variability and overall glucose load on CVD risk by monitoring blood glucose and pressure continuously for 24 h in patients with optimal glycemic control. Glucose fluctuations appear to activate the oxidative stress pathway, but cIMT is affected by chronic and postprandial hyperglycemia rather than glucose variability. The prognostic information of postprandial glucose in CVD was previously suggested by the DECODE Study group[18].

In theory, metabolic control could potentially attenuate or reduce cIMT. Twelve to fourteen weeks after treatment with the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, pioglitazone, cIMT was found to be significantly reduced. This effect was independent of improved glycemic control[19]. Pioglitazone is superior to glimepiride in terms of insulin resistance (IR) improvement and cIMT reduction [19]. PPAR-γ activation has both antiatherogenic and proatherogenic properties.

The effect of sitagliptin on cIMT has been reported from several studies. In the PROLOGUE study, sitagliptin was not superior to conventional treatment in terms of cIMT progression, despite significant improvement of glycemic control[20]. Alogliptin, a dipeptidyl peptidase-4 inhibitor (DDP-4i), was also found to attenuate cIMT progression[21]. It must be noted, though, that in the PROLOGUE trial, insulin-treated patients were excluded, and the HbA1c changes were lower compared to that in other studies.

Last but not least, cIMT progression is inhibited after metformin use. Metformin has several metabolic effects. It modulates hepatic glucose, improves IR and has recently been found to decrease the plasma DDP-4 activity with subsequent increase in glucagon-like peptide-1 (GLP-1) concentrations[22]. In T2DM without former CVD, the combination of liraglutide, a GLP-1 analogue, with metformin decreased cIMT after 8 mo. These changes could not be attributed entirely to the HbA1c improvement or lipid changes, suggesting a possible beneficial role in reducing plaque formation and inflammation, as previously reported[23]. Interestingly, the addition of metformin to insulin treatment, aiming at achievement of HbA1c < 7%, did not reduce cIMT in the Copenhagen Insulin and Metformin Therapy (CIMT) trial, a fact attributed partially to the smaller-than-expected final study size[24].

Table 1 summarizes the interventional and observational studies on cIMT outcomes after glycemic control in T2DM patients.

CAC score

The CAC score (CACS) is a well-established marker for the assessment of CVD risk in the general population.

Several researchers have reported CAC progression in T2DM. In a subanalysis of the Multiethnic Study of Atherosclerosis involving 5662 patients with T2DM or metabolic syndrome (MetS) without evident CVD, both categories were found to have greater incidence and accelerated progression of CAC compared to healthy individuals, which in turn can predict future CVD events[25].

Table 1 Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and carotid intima media thickness outcomes

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Nambi *et al*[10]	2010	Glucose levels 105 ± 30.7 mg/dL	Population-based cohort	Risk prediction model: Whether cIMT and plaque improves CHD risk prediction when added to traditional risk factors	13145	0.07 mm greater cIMT in the presence of DM
Kawasumi *et al*[15]	2006	5.8-6.4	Cohort	Insulin, sulfonylureas, nateglinide, metformin, pioglitazone, α-GI for 3 yr	100	HbA1c improvement > 0.2% prevents cIMT increase
Di Pino *et al* [14]	2014	5.7-6.4 or > 6.5	Cohort	Subjects without a previous history of diabetes were stratified into three groups according to HbA1c levels	274	Impaired cIMT even in pre-diabetes
Sharma and Pandita[16]	2017	> 7 or < 7	Cohort	T2DM duration > 1 yr or newly diagnosed, age 10-25 yr	45	HbA1c and longer diabetes duration affect cIMT
Di Flaviani *et al*[17]	2011	6.7 ± 1.3	Cohort	Continuous glucose monitoring; Diet and/or metformin	26	No association was observed between cIMT any glucose variability or overall glycemic load
Langenfeld *et al*[19]	2005	7.5 ± 0.9	RCT	Pioglitazone 45 mg/d *vs* glimepiride 2.7 ± 1.6 mg/d for 12-24 wk	173	Pioglitazone reduces cIMT independently of improvement in glycemic control
Oyama *et al* [20]	2016	6.2 < HbA1c < 9.4%	Multicenter PROBE	Sitagliptin 25 to 100 mg/d *vs* conventional treatment over 2 yr	442	Sitagliptin had no additional effect on cIMT progression
Rizzo *et al*[23]	2014	8.4 ± 0.8	Prospective pilot	Liraglutide added on metformin over 8 mo	64	Beneficial role in plaque formation and inflammation

α-GI: Alpha-glucosidase inhibitors; CHD: Coronary heart disease; cIMT: Carotid intima media thickness; CVD: Cardiovascular disease; DM: Diabetes mellitus; LDL: Low-density lipoprotein; RCT: Randomized controlled trial; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

The link between CAC and HbA1c or plasma glucose levels is well established. Anand *et al*[26] showed that suboptimal HbA1c levels (> 7%) are associated with increased risk for CAC progression. In an asymptomatic Korean population without T2DM, higher HbA1c levels predicted CAC, with the association being more prevalent in women[27]. The ARIC study showed higher relative risk for coronary heart disease for the highest quantile of HbA1c[28].

Although data for prediabetes are inconsistent, it is suggested that even without glycemic transition from impaired fasting glucose to T2DM, in the presence of IR, higher CAC prevalence is observed[29].

Moreover, symptomatic CAD patients (angina) with T2DM and poor glycemic control appear to have higher plaque volume[30]. The presence of noncalcified plaques and higher plaque burden are confirmed in asymptomatic T2DM patients as well[31, 32]. This underlies the importance of extended screening, even at the onset of diabetes.

A recent study from Germany showed that in established T2DM, poor glycemic control is associated with CAC progression. This progression is inevitable and rather unaffected by the burden of risk factors[33]. The question of whether tight glycemic control exerts beneficial effects on CAC, either regression or attenuation, remains unanswered because, even now, very few data are available. A small study from Schindler *et al*[34] showed that, after 1 year of treatment, effective glycemic control defined as fasting plasma glucose ≤ 126 mg/dL resulted in lower progression of both cIMT and CACS in treatment-naive, relatively newly-diagnosed T2DM patients (*i.e.* mean DM duration of 25 mo) without known CVD.

In the Veterans Affairs Diabetes (VADT) trial, after a 7.5-year follow-up period, intensive glucose lowering reduced cardiovascular events in patients with less extensive calcified coronary atherosclerosis, implying that aggressive glycemic control may be less effective in more advanced atherosclerosis. This was the case in other major trials such as the ACCORD study, which showed that patients without CVD and HbA1c < 8% are the ones who benefit the most from intensive treatment[35]. However, the extended follow-up to the VADT trial revealed that intensive glycemic control in patients with T2DM of > 5 years duration with previous cardiovascular events resulted in 8.6-fold fewer major cardiovascular events *per* 1000 person-years than those assigned to standard therapy[36]. This might suggest that a longer observation period is needed so that the beneficial effect becomes clinically apparent.

Yang *et al*[37] showed that long-term HBA1c variability plays an important role because metabolic stabilization for longer periods and at earlier stages of the disease might prevent subclinical coronary atherosclerosis. This is in agreement with the presence of the so-called legacy effect, a hypothesis that supports that early metabolic control has beneficial effects in terms of CVD prevention[37,38]. Recently, it has been shown that in asymptomatic CAD patients with known T2DM, optimal glycemic control attenuates CAC progression, whereas those patients with more calcified coronary lesions (defined as CAC > 400) appear to benefit the most.

At the tissue level, advanced end-glycation products were found to accelerate calcification in microvascular pericytes[39]. Other experimental data suggest a positive feedback loop of calcification and inflammation that plays an important role in disease progression, induced by the very same atherosclerotic lesions[40]. It is possible, though, that intensive treatment might be able to stop this vicious cycle triggered by baseline calcification.

Funck *et al*[41] showed higher burden and a greater number of atherosclerotic plaques in asymptomatic T2DM patients compared to healthy controls, despite optimal control of classical risk factors (hyperglycemia, BP, hyperlipidemia). They hypothesized that intensive risk factor control could not adequately control athero-sclerosis progression, probably due to higher burden at baseline, in accordance with data supporting higher CVD risk in T2DM patients with known macrovascular complications.

Malik *et al*[42] found that CAC score could improve long-term risk stratification to prevent CVD in T2DM and MetS. Importantly, CACS of 0 is associated with lower CVD risk independent of T2DM duration, glycemic control or insulin treatment. Even in T2DM patients with disease duration of more than 10 years, the absence of CACS was associated with low risk for future events, as in those with shorter disease duration. Finally, a recent study by Razavi *et al*[43] found that long-term absence of CAC during a follow-up period of 10 years in patients with T2DM and MetS was associated with baseline CAC of 0. Optimal multifactorial control is needed for healthy arterial aging. These data suggest that although T2DM is a CVD risk equivalent, it demonstrates considerable heterogeneity[44].

Table 2 summarizes the effect of glycemic control on CAC in T2DM patients.

FUNCTIONAL MARKERS

Flow-mediated dilatation

Originally described in 1992, Flow-mediated dilatation (FMD) is a noninvasive functional marker of subclinical atherosclerosis that utilizes ultrasound to record the reaction of brachial artery to an ischemic stimulus. It describes the vascular response to elevated blood flow, which is mediated by the produced vasoactive nitric oxide. FMD has been found to correlate with the severity and extent of coronary atherosclerosis [45].

In T2DM, postprandial hyperglycemia occurs early in the course of the disease and is thought to be a better marker of glycemic burden regarding associated complic-ations[46].

In a study with 30 T2DM patients, the investigators measured FMD and circulating endothelial cells (CECs), a marker of vascular damage. Patients were found to have impaired endothelial function compared to healthy controls, while HbA1c > 7% was associated with higher levels of CECs and lower FMD, suggesting the crucial role of glycemic control in diabetes management[47]. Some data suggest that glycemic control may result in improved vasodilatory responses and that certain glucose-lowering agents can improve FMD.

Watanabe *et al*[48] hypothesized that improvement of IR, a major metabolic cause of atherosclerosis, following treatment with troglitazone for 4 wk would improve endothelial dysfunction as well. At the end of the study, improvements in fasting glucose, insulin levels and FMD were documented. Similar results were confirmed in recent-onset diabetes without macrovascular complications, meaning that improvement of fasting insulin concentrations might be the underlying mechanism [49].

It seems that PPAR-γ agonists exert their antiatherogenic effect independently of their glucose-lowering effects, given that pioglitazone improves FMD irrespective of significant changes in insulin, C-reactive protein (CRP), free fatty acids, and adiponectin levels[50]. In nondiabetic patients with IR and recent history of stroke or transient ischemic attack, pioglitazone appears to reduce the risk for future CVD

Table 2 Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and coronary artery calcification outcomes

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Razavi *et al* [43]	2021	Fasting glucose > 126 mg/dL	Multiethnic cohort	Two CAC scans with a 10-yr interval	574	More than 40% of adults with MetS or T2DM and baseline CAC = 0 had long-term absence of CAC
Schindler *et al*[34]	2009	9.8 ± 2.7	Prospective	Glyburide 10-20 mg/d ± metformin 500-1000 mg/d; Observation for 14 ± 2 mo	39	Lower progression of cIMT and CAC with glucose-lowering treatment
Won *et al* [38]	2018	7.5 ± 1.2 and 6.4 ± 0.9	Retrospective, single-ethnicity, multicenter observational	Data on the impact of optimal glycemic control on CAC progression	1637	Attenuation of CAC progression, especially if CAC > 400
Funck *et al* [41]	2017	6.5 ± 0.7	Prospective cohort	Observational, 5-yr follow-up	106	CAC progression in DM compared to healthy. Independently associated with PWV
Malik *et al* [42]	2017	HbA1c measurements were not available at baseline	Prospective cohort	Observational	6814	Baseline CAC values most important progression determinant

CAC: Coronary artery calcification; cIMT: Carotid intima media thickness; DM: Diabetes mellitus; MetS: Metabolic syndrome; PWV: Pulse wave velocity; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

events[51]. The important role of reduction in IR is more apparent after comparison with insulinotropic sulfonylureas that achieve similar HbA1c effects without improvement of FMD[52,53]. The PROactive study did not shed light on the mechanism by which pioglitazone exerts its vascular benefit; it did, however, show a reduction in primary cardiovascular outcomes by almost 16%[54]. The reported improvement in fat cell metabolism is another argument toward the positive effects of thiazolidinediones beyond glycemic control[55]. Interestingly, gliclazide, unlike glimepiride, is reported to improve IR, CECs and FMD in a small group of T2DM patients[56,57].

FMD, glucose, and insulin levels were recorded prior to and after a dietary tolerance test in 30 newly diagnosed T2DM patients after treatment with acarbose (300 mg/d), nateglinide (270 mg/d), or placebo for 12 wk. Fasting FMD responses remained similar at follow-up in all groups. Despite comparable improvement in glycemic control, treatment with acarbose, unlike insulinotropic nateglinide, was associated with higher postprandial FMD responses. Major-Pedersen *et al*[58] reported that single administration of nateglinide initially improves postprandial endothelial dysfunction, but the effect disappears after 12 wk. This might imply that insulin secretion in the long term obviates the beneficial effects of controlled postprandial hyperglycemia on endothelial dysfunction.

Naka *et al*[59] compared the effect of two insulin sensitizers, metformin and pioglitazone, on poorly controlled T2DM already treated with sulfonylureas. Despite similar improvements in glycemic control, homeostatic model assessment insulin resistance index (HOMA-IR) and changes in FMD, only in the pioglitazone group did FMD and IR improve significantly. The authors concluded that treatment-induced changes in FMD are not associated with the effects on glycemic control or IR. Nevertheless, the reduction in IR achieved in this study was smaller compared to others, while the additional role of longer diabetes duration cannot be ignored.

Alpha-glucosidase inhibitors are also superior to nateglinide in terms of FMD improvement, IR index and markers of atherogenic dyslipidemia, despite similar HbA1c reduction[60].

Incretin-based treatments have been available for over a decade, and there is now evidence of important effects on cardiovascular outcomes beyond their glucose-lowering effects, such as antiatherogenic properties, modulation of arterial inflammation and endothelial function[61].

Improvement of both HbA1c and FMD in T2DM with stable CAD is reported after infusion of recombinant GLP-1. Interestingly, IR, as assessed by the hyperinsulinemic isoglycemic clamp technique, was not improved. Therefore, it is rather unlikely that GLP-1 exerts its beneficial effects on endothelium through improvement of insulin sensitivity index[62].

The beneficial effects of GLP-1 analogue treatment are possibly the result of improved insulin secretion and effect on postprandial glycemic control[63]. Based on this observation, Irace *et al*[64] tested the differences between intensification of metformin treatment with exenatide *vs* glimepiride and found that this combination ameliorates FMD through improvements of glycemic control and glycemic variability. The role of glucose variability and its possible deleterious effects on vascular endothelium were suggested earlier because glucose swings appeared to be more damaging for the endothelium than constantly high glucose levels[65]. Further large-scale trials are needed to establish the validity of this notion.

A randomized controlled trial comparing the effects of insulin glargine and of the GLP-1 analogue liraglutide failed to show an improvement in FMD after 14 wk, although liraglutide appeared to protect β-cell function and reduce oxidative stress. Low plasma concentration of liraglutide, the last dose of which was given 1 d prior to FMD measurement, might explain the negative results[66]. A similar conclusion regarding FMD is drawn after comparison of sitagliptin with glimepiride[67], which resulted in similar HbA1c improvements. That said, in a study from Egypt, sitagliptin improved endothelial dysfunction, insulin sensitivity, BP and hyperlipidemia in newly diagnosed T2DM patients[68]. A beneficial effect on FMD after sitagliptin is suggested by other authors without superiority against the α-glucosidase inhibitor voglibose[69].

More recently, Lambadiari *et al*[70] showed that in patients with poorly controlled T2DM, treatment intensification with incretin-based treatment improves not only FMD but other markers of subclinical atherosclerosis and cardiac function as well. This improvement is more profound in patients who achieve optimal glycemic control.

Linagliptin (at a dose of 5 mg daily) does not improve large-vessel endothelial function despite decreasing inflammation in patients with longer T2DM duration after 12 wk of treatment. Mitochondrial function and muscle oxygenation were not increased either[71]. This suggests that diabetes duration might play an additional, important role. Surprisingly, Ayaori *et al*[72] showed a deterioration of FMD after both alogliptin and sitagliptin. This was rather unexpected because positive effects had been reported, as mentioned earlier. A possible explanation for this phenomenon is that DPP-4 enzyme physiologically degrades GLP-1 (7-36) into the non-insulinotropic GLP-1 (9-36), which might be inactive but possibly exerts nitric oxide-mediated vasodilatory effects translated into worse FMD values.

In summary, studies with incretin-based agents are mostly relatively small, nonrandomized trials, and therefore observed differences on vascular function with the various agents are difficult to interpret.

The newest class of glucose-lowering agents, sodium-glucose co-transporter-2 (SGLT2) inhibitors, have been shown to reduce cardiovascular mortality in patients with T2DM. Several scholars have investigated whether glycemic control with these agents has any beneficial effects on the progression of atherosclerosis. In poorly controlled T2DM with CAD, administration of 100 mg canagliflozin for 4 wk improved HbA1c [from 9.2 (mean ± SD) ± 1.4 to 8.6 ± 1.1%, $P < 0.01$] and FMD[73]. In this study, none of the patients were treated with insulin.

Improvement of HbA1c and FMD was found in two recent studies after treatment with dapagliflozin. The first study included newly diagnosed metformin-treated T2DM patients with HbA1c of < 8%[74], whereas the second included T2DM patients with established ischemic heart disease. In the latter, surrogate markers of endothelial dysfunction and inflammation, such as adhesion molecule 1, endothelial nitric oxide synthase and high-sensitivity CRP, decreased with dapagliflozin therapy, and FMD correlated negatively with HbA1c[75]. Assessment of the Dapagliflozin Effect on Diabetic Endothelial Dysfunction of the Brachial Artery (ADDENDABHS2) trial, in which patients with poor glycemic control were randomized to receive either dapagliflozin or glibenclamide, shed more light on the effect of this agent[76].

A Mediterranean diet, except for reducing acute hyperglycemia and increasing antioxidant defenses and the protective action of GLP-1, improves FMD as well. Similarly, insulin sensitivity and FMD improves in T2DM patients with training, especially interval aerobic exercise[77].

Glycemic variability is believed to have unfavorable effects on macro- and microvascular events as well as all-cause mortality in T2DM. Wei *et al*[78] showed that glycemic visit-to-visit variability correlates with impairment of renal and endothelial dysfunction, as assessed by FMD.

In an attempt to elucidate the underlying mechanism, Costantino *et al*[79] examined the role of glycemic variability and mitochondrial oxidative stress in endothelial dysfunction. The investigators showed that glucose fluctuations rather than HbA1c cause epigenetic changes. This chromatin remodeling favors a proatherosclerotic phenotype. Owing to overexpression of stress molecules, FMD impairment and

oxidative stress persist even after improvement of HbA1c.

Table 3 summarizes interventional and observational studies on FMD outcomes after glycemic control in T2DM patients.

Pulse wave velocity

Pulse wave velocity (PWV) is a marker of AS, which in turn expresses the reduced flexibility and elasticity of blood vessels. It is assessed noninvasively and predicts future cardiovascular events and all-cause mortality[80]. Reduced elasticity occurs naturally with increasing age or under the rather destructive effect of metabolic disorders, such as T2DM or hypertension. T2DM and hypertension seem to have a synergistic effect on the progression of AS. PWV is a useful marker in the investigation of hypertension[81].

The prognostic value of PWV in T2DM is the subject of several studies. It has been estimated that for every 1 m/s increase in PWV, there is a 14.5% increase in the risk of CVD in patients with T2DM[82]. The association of glycemic control with PWV was earlier supported by Yokoyama *et al*[83], who showed that along with conventional risk factors, such as hyperlipidemia, hypertension and age, microalbuminuria is a determinant of cIMT and PWV and should, therefore, be taken into account for the detection of subclinical atherosclerosis and treatment stratification.

Control of hyperglycemia, even in the short term, appears to improve AS[83]. De Pascale *et al*[84] showed that good glycemic control is associated with lower AS and increased number of endothelial progenitor cells, which reflects the endothelium's regenerating capacity after damage.

Another study with 1675 participants in rural Brazil showed that increase of HbA1c by 1% was associated with increase of 54% in the odds of increased AS in the diabetic group. Both HbA1c and fasting blood glucose (FBG) had higher discriminatory power in the risk assessment for increased AS in nondiabetics compared to diabetics. Therefore, HbA1c elevation, even within the normal range, might cause endothelial dysfunction[85]. Similarly, Lee *et al*[86] concluded that even in the nondiabetic population, higher HbA1c levels were associated with increased brachial-ankle PWV. Therefore, early detection and management are essential to avoid atherosclerosis progression. There are additional studies that support the prognostic value of PWV in both diabetes and IGT[87].

In an older study, Webb *et al*[88] aimed to investigate the impact of glucose metabolism and IR on PWV. For this purpose, they enrolled 570 participants in the large ADDITION-Leicester program, who were divided into the three groups after a standard 75-g oral glucose tolerance test, namely NGT, IGT and T2DM. HbA1c as well as PWV gradually worsened from NGT to IGT and T2DM (all $P < 0.01$). Multivariate models demonstrated a strong relationship among PWV, fasting and 2-h postprandial glucose levels as well as HOMA-IR. Moreover, although all three indices contribute to PWV increase of about 3%–6%, postprandial glucose appears to be the most significant determinant. The effect of postprandial glucose on PWV was later supported by Li *et al* [89], who found that AS was increased in patients with IGT and newly diagnosed T2DM but not in those with impaired fasting glucose tolerance. Again, the hypothesis was that glycemic control, especially by targeting postprandial hyperglycemia, might reverse this phenomenon or even improve PWV.

Improvement of glycemic control after glimepiride, unlike glibenclamide, is associated with improved brachial ankle PWV and Augmentation Index (AIx). Notably, glimepiride decreases proinflammatory markers such as tumor necrosis factor-α, interleukin and CRP, with improvement of IR[90]. In that study, insulin-treated T2DM patients were used as the control group.

Beneficial effects on PWV through normalization of IR, as well as reduction of inflammation, are reported after glycemic control with rosiglitazone even in patients with established CAD[91].

The effect of hyperinsulinemia on other markers of subclinical atherosclerosis has already been discussed. In patients without evident macrovascular disease, higher insulin levels remain a significant predictor of PWV, even after adjustment for other well-established risk factors, concerning AS[92]. Interestingly, in this study, only 2% of the participants had a history of diabetes[92].

Another argument toward this hypothesis is that the use of insulin-sensitizer metformin in patients with nonalcoholic fatty liver disease (NAFLD)—a condition associated with IR—was associated with a significant reduction of both PWV and AIx in this population, with marginal improvements in fasting glucose, triglycerides, alkaline phosphatase and high-density lipoprotein cholesterol. These favorable effects of metformin were also observed in NAFLD patients without T2DM[93].

Table 3 Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and flow-mediated dilatation outcomes

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Watanabe *et al*[48]	2000	Fasting glucose 4.9 ± 0.3 mmol/L	Prospective cohort	Troglitazone 400 mg/d for 4 wk in non-DM	13	Improvement on fasting glucose, insulin and FMD
Caballero *et al*[49]	2003	7.5 ± 1.2 to 7.9 ± 1.5	Prospective randomized double-blinded	Troglitazone 600 mg/d for 12 wk	87	Improvement of FMD in newly diagnosed without CAD
Martens *et al* [50]	2005	7.1 ± 0.3	Prospective, randomized, crossover, placebo-controlled, double-blinded	Pioglitazone 30 mg/d for 4 wk	20	Improvement of FMD and adiponectin levels
Asnani *et al* [52]	2006	10 ± 2.3	Prospective randomized double-blinded	Pioglitazone 30 mg/d for 16 wk	20	Improvement of FMD
Chen *et al* [56]	2011	7.4 ± 1.3	Prospective controlled	Gliclazide 30-90 mg/d for 12 wk	58	Improvement of FMD, ECs and insulin resistance
Naka *et al* [59]	2012	7.8 ± 0.9 and 8.1 ± 1.3	Open-label randomized	Pioglitazone 30 mg/d or metformin 850 mg/d added to sulfonylureas for 6 mo	36	Improvement of FMD and insulin resistance
Sawada *et al* [60]	2014	6.9 ± 0.7 *vs* 7.0 ± 0.4	Randomized prospective	Miglitol 150 mg/d or nateglinide 270 mg/d for 16 wk	104	Improvement of FMD, insulin resistance index and markers of atherogenic dyslipidemia in the α-GI miglitol group
Irace *et al*[64]	2013	8.9 ± 1.2 and 8.2 ± 1.2	Observational	Exenatide 10-20 µg/d plus metformin *vs* glimepiride 2-4 mg/d plus metformin for 16 wk	20	Improvement of FMD; Better control on glycemic variability
Nomoto *et al* [66]	2015	8.6 ± 0.8 and 8.7 ± 0.8	Multicenter, prospective randomized parallel-group comparison	Liraglutide 0.3-0.9 mg/d *vs* glargine added on metformin and/or sulfonylurea for 14 wk	31	Similar FMD changes and β-cell function protection
Amira *et al* [68]	2017	Median (range): 8.7 (8.03 – 9.15)	Prospective controlled	Sitagliptin 100 mg/d for 24 wk	80	Improvement of FMD, insulin sensitivity blood pressure and hyperlipidemia
Kubota *et al* [69]	2012	7.3 ± 0.8	Open-labeled prospective observational single-arm	Sitagliptin 50 mg/d for 12 wk	40	Improvement of FMD and plasma adiponectin increase
Lambadiari *et al*[70]	2019	8.9 ± 1.8	Prospective cohort	Incretin-based treatment	100	Improvement of FMD and subclinical atherosclerosis after optimal glycemic control
Baltzis *et al* [71]	2016	7.1 ± 0.8	Randomized, double-blind, placebo-controlled	Linagliptin 5 mg/d *vs* placebo for 12 wk	40	No improvement in large vessel endothelial function
Takase *et al* [73]	2018	9.2 ± 1.4	Retrospective preliminary cross-sectional single-center pilot	Canagliflozin 100 mg/d for 4 wk	11	FMD improvement
Shigiyama *et al*[74]	2017	6.8 ± 0.5 and 6.9 ± 0.5	Prospective, randomized, open-label, blinded end-point, parallel-group, comparative	Dapagliflozin 5 mg/d added on metformin 1500 mg/d for 16 wk	80	Improvement of FMD in newly diagnosed T2DM
Zainordin *et al*[75]	2020	9.7 ± 1.9	Prospective, randomized, crossover, placebo-controlled, double-blind	Dapagliflozin 10 mg/d *vs* placebo added on metformin and insulin over 12 wk	81	No difference in FMD between the two groups observed; Significant reduction in surrogate marker of the endothelial function ICAM-1

α-GI: Alpha-glucosidase inhibitor; CAD: Coronary artery disease; DM: Diabetes mellitus; EC: Endothelial cell; FMD: flow-mediated dilatation; SD: Standard deviation; T2DM: Type 2 diabetes mellitus; IL: Interleukin.

In obese patients, treatment with metformin, rosiglitane or a combination of both with lifestyle modification improved not only glycemic control but PWV as well. In this study, however, only femoral PWV, known to assess peripheral stiffness rather than central AS, was associated with HbA1c[94]. On the contrary, reduction in the body fat mass of obese T2DM patients after exenatide improved lipid profile and aortic PWV, whereas PWV of the extremities did not change. The authors concluded that visceral fat reduction affecting the measurements could explain these findings

[95]. Using a different method, weight loss was found to strongly and independently reduce AS[96]. Because several classes of glucose-lowering agents are associated with weight gain, this should be considered prior to treatment stratification. Nevertheless, the beneficial effects of exenatide on PWV do not rely solely on weight reduction because improvements were shown after HbA1c reduction in T2DM patients without evident CAD, treated with metformin (sulfonylurea was prescribed in 5 patients only) [97].

Regarding the effects of DDP-4i on PWV, data are variable. When added to metformin, in T2DM with suboptimal HbA1c (> 7%), neither sitagliptin nor glibenclamide demonstrated any PWV benefits. Neither drug significantly influenced oxidative stress[98]. Zografou *et al*[99] showed no increase of PWV in drug-naive patients with T2DM, despite changes in HbA1c after treatment with vildagliptin. These patients had suboptimal HbA1c (7%–9%) at baseline and, moreover, a significant improvement in 24-h BP or waist circumference, all of which are important in terms of endothelial dysfunction.

Conversely, Duvnjak and Blaslov[100] studied 51 T2DM patients with good glycemic control, assigned to receive either sitagliptin or vildagliptin (100 mg/d). Both drugs were associated with improved PWV and AIx despite insignificant HbA1c changes. The authors concluded that the positive effects on AS are beyond glucose control. Favorable effects of linagliptin treatment for 26 wk with minimal yet significant HbA1c reduction (-0.4%, $P < 0.001$) were recently reported in newly diagnosed T2DM patients. Interestingly, after a 4-wk washout period, PWV returned to pre-intervention levels[101].

Large trials on DDP4-is support a neutral effect on CVD outcomes, although data show an important positive effect on PWV[102]. It is, therefore, possible that not all agents of this class share the same beneficial effects. Additionally, these studies differ in terms of baseline HbA1c as well as diabetes duration.

It has been proposed that a HbA1c of > 7% and diabetes duration of > 5 years are important cutoffs, above which stiffening of the arteries accelerates, especially in the presence of hypertension[103]. A recent observational study from Chang *et al*[104] rather confirms this observation. In hypertensive, poorly controlled T2DM (HbA1c³ 9%), reduction of HbA1c was not accompanied by significant differences in PWV or AIx in the short term. In a subanalysis based on cutoff HbA1c level of 7%, those with better HbA1c values had lower PWV, yet not significantly, and shorter T2DM duration. Even in high-risk middle-aged to elderly patients, PWV can be attenuated after improvements of glycemic control, BP, and heart rate. Furthermore, the rate of HbA1c reduction is associated with reduced risk for increased PWV[105].

Based on the aforementioned facts, it would be reasonable to hypothesize that improvement of glycemic and systemic BP control would attenuate or even improve PWV in T2DM. Amongst the various antidiabetic agents, SGLT2 inhibitors may efficiently lower both HbA1c and BP. Available data suggest that the reduction in CVD mortality is beyond their glycemic and antihypertensive effects, both factors being important for AS. Animal models show improvement in AS after the use of empagliflozin by promoting glycosuria and reducing systemic and renal AS based on improvements of periarterial and tubulointerstitial fibrosis[106]. Given that numerous studies support the relationship among AS, albuminuria and kidney injury[107], this could be a possible additional mechanism beyond glycemic control. In animal models, dapafliflozin improves hyperglycemia, AS and smooth muscle cell function, meaning that the positive effects on CVD mortality in diabetes are possibly owing to improvements in generalized vascular function[86]. The newest agent in this category, tofogliflozin, attenuated PWV in T2DM without history of CVD[108]. A recent metanalysis on newer agents showed that both GLP-1 analogues as well as DDP-4i effectively reduce PWV[109].

An association between HbA1c and PWV has been previously reported from some cross-sectional studies[110]. The implication that hyperglycemia alters material within the arterial wall and contributes to atherosclerosis was reinforced by other similar data. As Ferreira *et al*[105] reported, early intervention aiming to improve glycemic control might at least partially affect PWV, probably before the structural alterations occur.

Table 4 summarizes interventional and observational studies on PWV outcomes after glycemic control in T2DM patients.

Large and small artery elasticity

Arterial elasticity is also a noninvasive measure for the assessment of cardiovascular risk. It reflects the extent of vascular injury owing to cardiovascular risk factors and allows risk stratification with considerable prognostic value[111].

Table 4 Interventional and observational studies on glycemic control in type 2 diabetes mellitus patients and pulse wave velocity outcomes

Ref.	Year	HbA1c (%), mean ± SD	Type of study	Intervention	Sample size	Main findings
Koshiba *et al* [90]	2006	7.8 ± 2.0 and 7.7 ± 1.9	Prospective, randomized	Glibenclamide followed by glimepiride for 28 wk *vs* continuous administration of glibenclamide *vs* insulin therapy	34	Improvement of PWV, AIx, IR in the glimepiride group
de Oliveira *et al*[85]	2015	5.6 ± 0.7 and 6.3 ± 1.1	Prospective cohort	Observational	1675	Higher HbA1c levels are associated with higher PWV
Yu *et al*[91]	2007	6.5 ± 0.2	Prospective, randomized	Rosiglitazone 4 mg/d for 12 wk in diabetic patients with CAD	123	Decrease in PWV
Sofer *et al*[93]	2011	Fasting glucose: 132 ± 51 mg/dL	Prospective, randomized, placebo-controlled, double-blind	Metformin in patients with NAFLD with or without T2DM/IFG for 4 mo	63	Decrease in PWV and AIx
Shah *et al*[94]	2018	7.7± 2.0	Subanalysis of an RCT	Obese patients with metformin *vs* metformin plus intensive lifestyle intervention *vs* metformin plus rosiglitazone for 7.6 yr post-randomization	453	PWV increased; Attenuation possible
Scalzo *et al* [97]	2017	7.3 ± 1.1	Prospective, randomized, placebo-controlled, double-blind	Exenatide 20 µg/d subcutaneously, 30-60 min prior to meals, for 3 mo	23	Decrease in PWV
Koren *et al* [98]	2012	Fasting glucose: 169 ± 12 mg/dL	Prospective, controlled, open labeled, crossover	Sitagliptin 100 mg/d or glibenclamide 5 mg/d for 3 mo, cross-over switch for an additional 3 mo	34	No PWV benefits; Beneficial BMI effects of sitagliptin
Zografou *et al* [99]	2015	8.1 ± 0.8	Prospective randomized open-label	Vildagliptin 100 mg/d plus metformin 1700 mg/d *vs* metformin monotherapy 1700 mg/d	64	No effect on arterial stiffness in drug-naive patients with T2DM
Duvnjak and Blaslov[100]	2016	6.9 ± 1.1	Prospective, uncontrolled, open label, parallel-arm, randomized	Sitagliptin 100 mg/d or vildagliptin 100 mg/d for 3 mo	51	Decrease in PWV and Aix; No HbA1c reduction
De Boer *et al* [101]	2017	6.3 ± 0.4	Prospective, randomized, placebo-controlled, double-blind	Linagliptin 5 mg/d *vs* placebo for 26 wk	45	PWV improvement disappears after 4-wk washout period in newly diagnosed T2DM
Chen *et al* [103]	2009	6.9 ± 1.3	Prospective cohort	Observational	1000	PWV correlates with HbA1c and diabetes duration in patients with T2DM and hypertension
Chang *et al* [104]	2018	11.7 ± 1.9	Prospective cohort	Insulin or oral hypoglycemic agents (metformin, sulfonylurea, α-GI, DDP-4i) or combined insulin and oral agents for 12 wk	64	No PWV improvement
Ferreira *et al* [105]	2015	7.6 ± 1.4	Prospective cohort	Metformin, sulfonylureas or insulin for 4.2 yr	417	Attenuation of PWV progression

α-GI: Alpha-glucosidase inhibitor; AIx: Augmentation Index; BMI: Body mass index; CAD: Coronary artery disease; cIMT: Carotid intima media thickness; DDP-4i: dipeptidyl peptidase-4 inhibitor; HCT: Hydrochlorothiazide; IFG: Impaired fasting glucose; IR: Insulin resistance; NAFLD: Nonalcoholic fatty liver disease; PWV: Pulse wave velocity; RCT: Randomized controlled trial; SD: Standard deviation; T2DM: Type 2 diabetes mellitus.

It is suggested that small arteries compared to large ones—that is, the aorta—contain a more repairable component regarding arterial elasticity, where fixed fibrotic tissue is more prominent and requires a longer repair period[112]. Interestingly, in a group of nondiabetic patients with CAD, only small (C2) and FMD appeared to be impaired compared to healthy controls, suggesting that C2 is a surrogate marker for the clinical evaluation of endothelial function[113].

Shargorodsky *et al*[114] showed that 6 mo of treatment with rosiglitazone in 52 patients with moderate CVD risk and poor glycemic control (longer disease duration of 5–28 years, 24% on insulin) resulted in impressive improvement of C2, which was attributed to improvements in IR and hyperinsulinemia. Though there was a tendency toward improvement for large (C1), it did not reach statistical significance. The authors mentioned that a longer follow-up period was probably needed. After an

extended follow-up period of 2 years, improvements in both C1 and C2 after rosiglitazone treatment were confirmed, and the beneficial effect deteriorated after treatment discontinuation. Moreover, this was independent of glycemic control, implying the central role of hyperinsulinemia in AS. Last but not least, with data supporting that rosiglitazone inhibits cIMT progression in nondiabetic individuals, the antiatherogenic effect of PPAR-γ activators is independent of glycemic control[115].

Prisant *et al*[116] aimed to investigate the relationship of HbA1c and arterial elasticity by performing measurements of both C1 and C2 in 111 subjects with longer diabetes duration (12 years) and poor glycemic control (HbA1c 8.9%). Increasing age and HbA1c were found to be associated with small, but not large, artery elasticity, whereas women with T2DM had lower C2 compared to men. In this study, 26% of participants were type 1 DM patients.

That said, McVeigh *et al*[117], in an older study analyzing intra-arterial brachial artery pulse waves in T2DM, suggested reduced C2 in T2DM but no correlations with fasting glucose or HbA1c, whereas C1 was not reduced. They used mostly diet intervention and/or sulfonylurea and metformin to achieve glycemic control.

Several data support that C2 can be raised by means of improvement of HbA1c such as through fish oil supplements[118] or telmisartan[119]. Mourot *et al*[120] investigated the effect of a cardiovascular rehabilitation program on arterial compliance in patients with T2DM and CAD. These patients had suboptimal or poor glycemic control at the time of admission. After 6 wk, improvement of arterial compliance was observed, probably thanks to regular exercise, optimal glucose-lowering, and hypolipidemic treatment. Because arterial compliance was improved in the subpopulation with no change in antihypertensive treatment as well, the observed increase extended beyond antihypertensive treatment. Moreover, decrease of IR through training and amelioration of glycemic control, which was achieved by insulin, oral agents, or a combination of both, appear to have contributed to this improvement as well.

CONCLUSION

The additional use of these noninvasive markers of atherosclerosis strengthens the predictive risk for developing CAD beyond traditional risk assessment and enables the monitoring of selected treatment in T2DM. Progression or even regression of atherosclerosis is possible in T2DM, especially in patients with newly diagnosed diabetes, with relatively good glycemic control and use of newer agents, such as DDP4-is and SGLT2 inhibitors. This is best reflected in the updated guidelines, which support their use after metformin treatment, which also has beneficial effects. A multifactorial intervention with improvement of classical risk factors, such as hypertension and BP, should always be considered. Both structural and functional markers are easily accessible and could be an additional tool for clinicians to screen high-risk patients, with CAC, cIMT and PWV showing less intra-observer variability compared to FMD and small artery elasticity index. Despite considerable evidence for predictive value especially for cIMT, CAC and PWV, larger studies and studies over longer periods are needed to correlate clinical outcomes with improvement of subclinical atherosclerosis.

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Basic Study

Profilin-1 is involved in macroangiopathy induced by advanced glycation end products *via* vascular remodeling and inflammation

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Abstract

BACKGROUND

The accumulation of advanced glycation end products (AGEs) have been implicated in the development and progression of diabetic vasculopathy. However, the role of profilin-1 as a multifunctional actin-binding protein in AGEs-induced atherosclerosis (AS) is largely unknown.

AIM

To explore the potential role of profilin-1 in the pathogenesis of AS induced by AGEs, particularly in relation to the Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) signaling pathway.

METHODS

Eighty-nine individuals undergoing coronary angiography were enrolled in the study. Plasma cytokine levels were detected using ELISA kits. Rat aortic vascular smooth muscle cells (RASMCs) were incubated with different compounds for different times. Cell proliferation was determined by performing the MTT assay and EdU staining. An AGEs-induced vascular remodeling model was established in rats and histological and immunohistochemical analyses were performed. The mRNA and protein levels were detected using real-time PCR and Western blot analysis, respectively. *In vivo*, shRNA transfection was performed to verify the role of profilin-1 in AGEs-induced proatherogenic mediator release and aortic remodeling. Statistical analyses were performed using SPSS 22.0 software.

RESULTS

Compared with the control group, plasma levels of profilin-1 and receptor for AGEs (RAGE) were significantly increased in patients with coronary artery disease, especially in those complicated with diabetes mellitus ($P < 0.01$). The levels of profilin-1 were positively correlated with the levels of RAGE ($P < 0.01$); additionally, the levels of both molecules were positively associated with the degree of coronary artery stenosis ($P < 0.01$). *In vivo*, tail vein injections of AGEs induced the release of proatherogenic mediators, such as asymmetric dimethylarginine, intercellular adhesion molecule-1, and the N-terminus of procollagen III peptide, concomitant with apparent aortic morphological changes and significantly upregulated expression of the profilin-1 mRNA and protein in the thoracic aorta ($P < 0.05$ or $P < 0.01$). Downregulation of profilin-1 expression with an shRNA significantly attenuated AGEs-induced proatherogenic mediator release ($P < 0.05$) and aortic remodeling. *In vitro*, incubation of vascular smooth muscle cells (VSMCs) with AGEs significantly promoted cell proliferation and upregulated the expression of the profilin-1 mRNA and protein ($P < 0.05$). AGEs (200 μg/mL, 24 h) significantly upregulated the expression of the STAT3 mRNA and protein and JAK2 protein, which was blocked by a JAK2 inhibitor (T3042-1) and/or STAT3 inhibitor (T6308-1) ($P < 0.05$). In addition, pretreatment with T3042-1 or T6308-1 significantly inhibited AGEs-induced RASMC proliferation ($P < 0.05$).

CONCLUSION

AGEs induce proatherogenic events such as VSMC proliferation, proatherogenic mediator release, and vascular remodeling, changes that can be attenuated by silencing profilin-1 expression. These results suggest a crucial role for profilin-1 in AGEs-induced vasculopathy.

Key Words: Advanced glycation end products; Profilin-1; Diabetic macroangiopathy; Atherosclerosis; Vascular remodeling; Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway

Core Tip: The formation and accumulation of advanced glycation end products (AGEs) contribute to accelerated macrovascular complications in individuals with diabetes. Currently, few studies have examined the role of profilin-1 in vasculopathy induced by AGEs. The present study proved that AGEs induced proatherogenic events such as vascular smooth muscle cell proliferation, proatherogenic mediator expression, and vascular remodeling, changes that were attenuated by silencing profilin-1 gene expression. These data suggested for the first time that profilin-1 was involved in AGEs-induced aortic atheroma formation in rats, indicating that drugs targeting profilin-1 may become a potential therapeutic strategy for ameliorating atherosclerosis secondary to diabetes.

INTRODUCTION

Diabetic macroangiopathy is the leading cause of morbidity and mortality in patients with diabetes. The pathophysiological basis of diabetic macroangiopathy is atherosclerosis (AS), a complicated biological process including endothelial dysfunction, macrophage accumulation, inflammatory factor infiltration, vascular smooth muscle cell (VSMC) proliferation, and intima-media remodeling[1], which ultimately leads to

vessel wall thickening and lumen stenosis. A large number of clinical studies have shown that the incidence of diabetic vascular complications has not decreased in patients with chronic long-term hyperglycemia, despite strict blood glucose and risk factor control[2,3]. Thus, the identification of effective new targets for the prevention and treatment of diabetic macroangiopathy is an urgent unsolved problem.

The production of advanced glycation end products (AGEs) is due to chronic hyperglycemia, aging, and chronic degenerative diseases[4]. The formation and accumulation of AGEs contribute to accelerated macrovascular complications in individuals with diabetes, and high serum levels of AGEs and receptor for AGEs (RAGE) are associated with an increased incidence and severity of coronary artery disease (CAD)[5-7]. According to previous studies, AGEs are involved in the development of AS through two major pathways: Binding to RAGE or cross-linking of proteins[8]. On the one hand, AGEs in the vascular wall cross-link extracellular matrix proteins, leading to arterial stiffness and atherosclerotic plaque formation[9]. On the other hand, the binding of AGEs to RAGE elicits the generation of reactive oxidative species (ROS), subsequently activating mitogen-activated protein kinase and nuclear factor kappa-B signaling and activating the pathways related to diabetic complications [10].

Profilin-1 is a well-known highly conserved and ubiquitously expressed multifunc-tional actin-binding protein (12 to 15 KD) that plays an essential role in regulating cytoskeletal rearrangement and redistribution by promoting actin polymerization and remodeling[11,12]. Based on accumulating evidence, profilin-1 levels are markedly increased in serum, the diabetic endothelium, and atherosclerotic lesions of patients with pathological conditions such as hypertension, AS, and diabetes[13-15]. Cardio-vascular risk factors such as oxidized low density lipoprotein (ox-LDL) and Ang II significantly upregulate the expression of profilin-1 in cultured endothelial cells or VSMCs, leading to endothelial dysfunction or VSMC proliferation[1,16,17], both of which contribute to AS. In addition, chronic vascular inflammation also contributes to the initiation and progression of atherosclerosis secondary to diabetes[18]. A growing body of evidence indicates important roles for profilin-1 in vascular remodeling and vascular inflammation. In spontaneously hypertensive rats, overexpression of profilin-1 significantly promotes aortic remodeling, including an increase in vessel size, wall thickness, and collagen content, partially through the p38-iNOS-peroxynitrite pathway [19]. *In vivo*, profilin-1 links the actin cytoskeleton and its dynamics directly to vascular inflammation, which is mediated by a series of inflammatory mediators[20]. As the role of profilin-1 in endothelial damage triggered by the diabetic milieu is surprisingly similar to that triggered by lipid oxidation[15] and attenuated expression of profilin-1 exerts protective effects on AS in LDL receptor-null mice[21], profilin-1 is expected to become a promising therapeutic target for preventing the diabetes complication of vascular injury[22].

As shown in our previous study, AGEs induce endothelial dysfunction and cardiomyocyte hypertrophy *in vitro*, changes that are attenuated by the knockdown of profilin-1 expression[12,23]. However, to date, few studies have examined the role of profilin-1 in vascular remodeling induced by AGEs. Thus, the present study aimed to explore the role of profilin-1 in AGEs-induced macroangiopathy and identify vascular remodeling- and inflammation-dependent mechanisms that might account for the atheroprotective effects of profilin-1 silencing on AS both *in vivo* and *in vitro* and to provide a theoretical basis for targeting profilin-1 in the therapy of the diabetes complication of AS.

MATERIALS AND METHODS

Chemicals and reagents

AGEs were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was supplied by Every Green Co. Ltd. (Hangzhou, Zhejiang Province, China). Asymmetric dimethylarginine (ADMA) and the anti-β-actin antibody were purchased from Sigma (St. Louis, MO, United States). Intercellular adhesion molecule-1 (ICAM-1) ELISA kits and Griess reagents were purchased from Jiancheng Biological Medical Engineering Institute (Nanjing, Jiangsu Province, China). N-terminal propeptide of type III procollagen (PIIINP) ELISA kits were supplied by Vscn Life Sciences, Inc. (Wuhan, Hubei Province, China). Phenylmethylsulfonyl fluoride (PMSF) and BCA protein kits were purchased from Beyotime Company (Jiangsu Province, China). The anti-profilin-1 antibody was obtained from Abcam (Shanghai, China). Western blotting kits and secondary IgG antibodies were purchased from KPL (Gaithersburg,

MD, United States). The primers were synthesized by Shanghai Sangong Co. (Shanghai, China). RT reagent kits were obtained from Thermo Scientific (Waltham, MA, United States). Profilin-1 shRNA adenovirus vectors (Ad-profilin-1 shRNA) and blank control adenovirus vectors (Ad-GFP) were constructed by Hanbio Co. (Shanghai, China).

Study population

Eighty-nine patients undergoing coronary angiography (CAG) at the Department of Cardiology of XiangYa Hospital of Central South University from September 2017 to March 2018 were enrolled. Groups were divided as follows: Control group (27 patients) with stenosis < 50% in all coronary vessels; CAD group (31 patients) with stenosis ≥ 50% in at least one vessel but without diabetes; and CAD + diabetes mellitus (DM) group (31 patients) with stenosis ≥ 50% in at least one vessel with diabetes. The diagnosis of type 2 diabetes was made according to the criteria of the American Diabetes Association reported in 2017[24]. Informed consent was obtained from enrolled individuals prior to participation in the study, and privacy rights were always observed. The present study was approved by the ethical review committee of Xiangya Hospital of Central South University.

Clinical data collection and CAG

Peripheral blood was collected from all patients, and the levels of biochemical factors, such as fasting plasma glucose, glycosylated serum protein, glycosylated hemoglobin, total cholesterol, low-density lipoprotein, high-density lipoprotein, triglyceride, serum creatinine, and uric acid, were measured using an automatic biochemical analyzer. Clinical data, including age, sex, weight, systolic blood pressure, diastolic blood pressure, and CAD course, were collected. Each patient underwent angiography of the left and right coronary arteries using a GE Innova 3000 angiography CAG examination through a radial artery puncture with a selective angiography catheter. Coronary artery disease was defined as ≥ 50% stenosis in major vessels (the left anterior descending artery, circumflex artery, and right coronary artery and their major branches). The severity of coronary artery stenosis was quantitatively assessed according to CAG using the Gensini scoring system.

Cell culture

Rat aortic vascular smooth muscle cells (RASMCs) were purchased from ATCC (Manassas, VA, United States) and placed in an incubator at 37 °C with 90% humidity and an atmosphere containing 5% CO_2. RASMCs were cultured in DMEM medium supplemented with 10% FBS (Gibco BRL, Grand Island, NY, United States), 100 U/mL penicillin, and 100 mg/mL streptomycin (Beyotime, China). After reaching 90% confluence, the cells were subcultured and incubated with different concentrations (0, 50, 100, 150, 200, or 400 µg/mL) of AGEs for different times (0, 24, 48, and 72 h) at passages 2-3 or incubated with the JAK2 inhibitor (T3042-1, 15 µmol/L) or STAT3 inhibitor (T6308-1, 5 µmol/L).

Cell proliferation assay

The viability and proliferation of VSMCs were determined using a MTT (5 mg/mL, Beyotime Company, Shanghai, China) assay according to the manufacturer's instructions. Briefly, RASMCs were seeded on 96-well plates at a density of 7×10^6 cells/mL in DMEM supplemented with 10% FBS. The medium was changed to DMEM containing 1% FBS to make them quiescent for 24 h, and then different concentrations of AGEs were added and cocultured with the cells. Finally, 20 µL of 5 mg/mL MTT was added to the culture medium and incubated for 4 h. The formazan crystals in each well were dissolved in 150 µL of DMSO, and the absorbance was evaluated at 595 nm using a microplate reader (PerkinElmer, United States) to assess cell proliferation.

RASMCs were seeded on 6-well plates at a density of 1×10^4 cells/mL. After reaching 60% confluence, proliferation was assessed according to the instructions of the EdU Staining Proliferation Kit (iFluor 647). Briefly, the EdU solution was added to RASMCs and incubated for 2-4 h. Then, fixative solution and permeabilization buffer were added successively. After incubation with the reaction mix to fluorescently label cells with EdU for 30 min, cell proliferation was detected and quantified using fluorescence microscopy and flow cytometry.

Establishment of an AGEs-induced vascular remodeling model in rats

AGEs were prepared using a previously reported method[16]. Briefly, BSA (10 g/L) was incubated in 0.2 M phosphate buffer (PBS, pH 7.4) with D-glucose (90 g/L)

containing 100 U/mL penicillin and 100 mg/mL streptomycin in the dark at 37 °C. After 12 wk of incubation, the preparations were dialyzed against PBS three times at 4 °C to remove free glucose for 24 h and then separated into aliquots and stored at -20 °C until use.

Sprague-Dawley (SD) rats were fed under conditions of a controlled temperature (22 ± 1 °C) and humidity (50%-60%). AGEs-BSA was injected into the tail veins at a dose of 25 mg/kg/d per rat for 0, 20, 40, and 60 d. Ad-profilin-1 shRNA or Ad-GFP was injected into the tail vein twice at a dose of 3 × 10⁹ PFU per injection with an interval of 4 wk between injections. The experimental protocols used in this study were approved by the Medicine Animal Welfare Committee of Xiangya Hospital, Central South University (Changsha, Hunan Province, China).

Animal preparation

One-month-old male SD rats were purchased from Slac Laboratory Co. (Shanghai, China) and randomly divided into different groups (n = 6) after 1 wk of acclimation: (1) Control group: SD rats were intravenously injected with normal saline; (2) AGEs group: SD rats were intravenously injected with AGEs-BSA for 20, 40, or 60 d; (3) AGEs-S group: SD rats were intravenously injected with AGEs-BSA for 60 d and Ad-profilin-1 shRNA; and (4) AGEs-V group: SD rats were intravenously injected with AGEs-BSA for 60 d and Ad-GFP.

All rats were anesthetized with pentobarbital sodium (1%), and blood samples were collected after decapitation and centrifuged for 10 min at 4 °C at a rate of 3000 *g*. Supernatants were stored at -80 °C until analysis. The thoracic aorta was separated and divided into three parts: (1) One part was fixed with 10% formalin for 24 h, sequentially dehydrated in 70%, 80%, 90%, and 100% ethanol, rendered transparent with xylene, embedded in paraffin wax blocks, and stored at 4 °C until hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC); (2) The second part was homogenized in TRIzol reagent for real-time PCR analysis; and (3) The third part was homogenized in RIPA lysis buffer and 0.1 mmol/L PMSF for Western blot analysis. All procedures were performed in compliance with guidelines from the Ethics Committee for Animal Care and Research at Xiangya Hospital of Central South University (Changsha, Hunan Province, China).

Histology and immunohistochemical staining

H&E staining was performed on 3 mm sections from formalin-fixed and paraffin-embedded thoracic aorta tissue using standard protocols. After H&E staining, the morphological changes in blood vessels were observed under a light microscope. An Image-Pro Plus computer pathological image analysis system was used to calculate the media thickness (MT), lumen diameter (LD), medial area (MA), lumen area (LA), and smooth muscle layers (SMLs) of the thoracic aorta. The median MT/LD and MA/LA indicated the degree of hyperplasia.

IHC was performed as previously described[25] to detect the expression of profilin-1. Briefly, sections of aortic tissues with a thickness of 3 mm were incubated overnight at 4 °C with the primary antibody against profilin-1 (1:50) and then incubated with a horseradish peroxidase-labeled goat anti-rabbit immunoglobulin-G secondary antibody (1:1000) at 37 °C for 20 min. After three washes with PBS, the samples were incubated with diaminobenzidine for coloration and counterstained with hematoxylin for 2 min to stain the nuclei. Photographs were captured under a high-power microscope (200 ×); positively stained cells were brown in color, and integral optical density values were measured using an NIS-Elements AR 3.0 pathological image analysis system.

RNA isolation and real-time PCR

Total RNA was extracted from cells grown in a 6-well plate using TRIzol reagent, and cDNA was synthesized from 1 μg of RNA using the First-Strand Synthesis System for PCR according to the manufacturer's instructions. The following primer pair was used to amplify profilin-1: Forward primer, 5'-TGACCTCATCTGTCCCTTCC-3'; reverse primer, 5'-ACAGGAGGGGGTATGGGTAG-3'. The following primer pair was used to amplify *GAPDH*: Forward primer, 5'-ACCCAGAAGACTGTGGATGG-3'; reverse primer, 5'-CACATTGGGGGTAGGAACAC-3'. The primer pair used for *JAK2* amplification was as follows: Forward primer, 5'-CGGAACACCTTGTCCTGAAT-3'; reverse primer, 5'-GAGTCAGCTGGGAAAAGCAC-3'. The primer pair used for *STAT3* amplification was as follows: Forward primer, 5'-TGATGCGCTCTTAT-GTGAGG-3'; reverse primer, 5'-GGCGGACAGAACATAGGTGT-3'. Amplification was performed with an initial incubation at 95 °C for 5 min, and 40 cycles of

denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s to detect profilin-1, *GAPDH, STAT3*, and *JAK2* expression. All amplification reactions for each sample were performed in triplicate, and data were analyzed using the 2$^{-\Delta\Delta CT}$ method. The mRNA levels of profilin-1, *STAT3*, and *JAK2* were normalized to the level of the *GAPDH* mRNA.

Western blot analysis

VSMCs were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and centrifuged at 12000 *g* at 4 °C for 15 min. The concentration of the total protein extract was measured using BCA protein assay kits. Approximately 60 µg of protein was separated on 15% SDS-PAGE gels and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, United States). The membranes were blocked with 5% fat-free milk in PBST buffer (0.1% Tween 20 in PBS) for 90 min and subsequently probed with primary antibodies against JAK2 (1:1000), STAT3 (1:2000), profilin-1 (1:1500), or β-actin (1:5000) at 4 °C overnight, followed by incubation with an HRP-conjugated IgG secondary antibody at room temperature for 1 h. The bands on the membranes were visualized by adding chemiluminescence reagent, and the gray value was then analyzed using ImageJ software v1.37c (National Institutes of Health, Bethesda, MD, United States).

shRNA transfection in vivo

The adenovirus interference vector Ad-profilin-1 shRNA was generated from pHBAd-U6-GFP using Gateway recombination technology. Briefly, Ad-profilin-1 shRNA was transfected into DH5a cells for adenovirus packaging and titer. The recombinant adenoviruses were measured using the immunization method. Primers for the profilin-1 shRNA were forward, 5'-TGCTGATTTCTTGTTGATCAAACCACGTTTTG-GCCACTGACTGACGTGGTTTGCAACAAGAAAT-3' and reverse, 5'-CCTGATTTCT-TGTTGCAAACCACGTCAGTCAGTGGCCAAAACGTGGTTTGAT-CAACAAGAAATC-3'. The Ad-profilin-1 shRNA adenovirus vector was injected into the tail vein of SD rats at a dose of 3 × 10⁹ PFU with an interval of 4 wk to silence the expression of profilin-1. The transfection efficiency in each experiment was determined by measuring profilin-1 protein expression using Western blot analysis.

Detection of plasma cytokine levels

The contents of ICAM-1 and PIIINP were detected using ELISA kits, and the concentration of ADMA was measured using high-performance liquid chromatography as previously described[26]. The standard curve for each assay was constructed in accordance with the manufacturer's provided sample. Each sample was assayed in duplicate.

Statistical analysis

Test data of each sample accorded with the normal distribution and are expressed as the mean ± SD. Statistical analyses were performed using SPSS 22.0 software (IBM, Chicago, IL, United States). The comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by the test of pairwise comparisons. Associations between profilin-1, RAGE, and Gensini scores were assessed by Pearson correlation analysis. A value of $P < 0.05$ was considered to be of statistical difference.

RESULTS

Baseline characteristics

Enrolled individuals with CAD and DM regularly received cardiovascular drug therapy, including antihypertensive and lipid-lowering drugs. The baseline characteristics of the subjects are presented in Table 1. None of the groups showed significant differences in age, percentage of males, SBP, DBP, LDL-cholesterol levels, high-density lipoprotein cholesterol levels, or serum uric acid levels ($P > 0.05$). Compared with the control group, the individuals in the CAD group and CAD + DM group had a higher weight and higher plasma levels of glycosylated hemoglobin (HbA1c), triglyceride (TG), total cholesterol (TC), high sensitivity C-reactive protein (hs-CRP), and blood urea nitrogen (BUN) ($P < 0.05$). Compared with the CAD group, those who developed CAD combined with DM showed higher plasma fasting plasma glucose (FPG), HbA1c, TG, and serum creatinine levels ($P < 0.05$).

Table 1 Comparison of clinical data (mean ± SD)

	Control group (*n* = 27)	CAD group (*n* = 31)	CAD+DM group (*n* = 31)
Age (yr)	60 ± 8	61 ± 9	64 ± 8
Sex (n)			
Male	14	15	17
Female	13	16	14
Weight (kg)	60.87 ± 7.67	67.15 ± 12.00[a]	65.55 ± 10.30[a]
SBP (mmHg)	123.54 ± 14.28	126.41 ± 16.56	127.35 ± 19.49
DBP (mmHg)	74.46 ± 6.66	75.71 ± 10.45	78.74 ± 9.57
FPG (mmol/L)	4.86 ± 0.56	5.47 ± 0.87	9.92 ± 3.26[a,c]
HbA1c (%)	4.90 ± 0.58	6.07 ± 0.93[a]	7.43 ± 1.70[a,c]
GSP (mmol/L)	1.81 ± 0.19	2.27 ± 0.39	2.78 ± 1.19[a,c]
TG (mmol/L)	1.21 ± 0.35	1.79 ± 1.25[a]	2.44 ± 2.15[a,c]
TC (mmol/L)	2.10 ± 1.61	4.40 ± 1.08[a]	4.51 ± 1.26[a]
LDL-C (mmol/L)	2.32 ± 0.53	2.63 ± 0.67	2.59 ± 1.22
HDL-C (mmol/L)	1.35 ± 0.31	2.48 ± 1.43	1.50 ± 1.14
hs-CRP (mg/L)	1.34 ± 0.61	3.84 ± 3.15[a]	4.65 ± 2.70[a]
BUN (mmol/L)	5.29 ± 0.73	5.53 ± 1.44[a]	5.85 ± 1.65[a]
SCr (μmol/L)	79.3 ± 15.2	82.3 ± 23.3	93.2 ± 21.4[a,c]
SUA (mmol/L)	309.2 ± 92.3	316.3 ± 81.7	327.6 ± 69.8

[a]*P* < 0.05 compared with the control group.

[c]*P* < 0.05 compared with the coronary artery disease group.

CAD: Coronary artery disease; DM: Diabetes mellitus; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; GSP: Glycosylated serum protein; TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; hsCRP: High sensitivity C-reactive protein; BUN: Serum urea nitrogen; Scr: Serum creatinine; SUA: Serum uric acid.

Relationships between profilin-1 expression, RAGE expression, and Gensini scores

The plasma concentrations of profilin-1 and RAGE in enrolled individuals were detected using ELISA kits. The results of one-way ANOVA showed significantly increased concentrations of profilin-1 and RAGE in the CAD and CAD + DM groups compared with the control group (*P* < 0.01) (Figure 1A and B). As expected, plasma profilin-1 and RAGE levels were significantly higher in patients with CAD and DM than in those with CAD alone (*P* < 0.01). A similar significant difference was also observed in Gensini scores (Figure 1C).

In the Pearson correlation analysis, both baseline profilin-1 and RAGE levels showed a strong significant positive correlation with Gensini scores (R^2 = 0.7494, *P* = 0.0005 and R^2 = 0.8424, *P* = 0.0010, respectively) (Figure 1D and E), and the plasma RAGE and profilin-1 levels were also positively correlated (R^2 = 0.7161, *P* = 0.0010) (Figure 1F).

### Morphological changes in the rat thoracic aorta induced by AGEs

After tail vein injections of AGEs (25 mg/kg/d) for different times, the histological morphology of the thoracic aorta in rats was assessed to investigate the effect of AGEs on the aortic structure. H&E staining (200 ×) showed significant vascular remodeling of the thoracic aorta in AGEs-injected rats, including thickened aortic walls, looser or even broken elastic fibers, and broadened endothelial gaps. Significant proliferation and a disordered arrangement of SMCs were also observed in the AGEs group, leading to lumen stenosis (Figure 2). We measured the MT, LD, ratio of MT/LD, MA, LA, ratio of MA/LA, and SMLs of the thoracic aorta. Compared with the control group, the injection of AGEs significantly increased the MT/LD and MA/LA ratios, which are considered signs of vascular remodeling (*P* < 0.01, Table 2). The number of SMLs also increased 40 d after the AGEs injection (*P* < 0.05, Table 2).

Table 2 Media thickness, lumen diameter, media thickness/lumen diameter, medial area, lumen area, medial area/lumen area, and smooth muscle layers of the thoracic aorta

	Control (*n* = 6)	AGEs-20 (*n* = 6)	AGEs-40 (*n* = 6)	AGEs-60 (*n* = 6)
MT (mm)	45.83 ± 3.54	57.17 ± 3.25[a]	74.83 ± 4.62[a]	79.33 ± 3.08[a]
LD (mm)	737.5 ± 9.89	769.83 ± 15.73[a]	862.33 ± 21.99[a]	857.67 ± 15.06[a]
MT/LD (%)	6.21 ± 0.41	7.42 ± 0.35[a]	8.67 ± 0.37[a]	9.25 ± 0.34[a]
MA (mm^2)	0.53 ± 0.01	0.54 ± 0.01[a]	0.55 ± 0.01[a]	0.56 ± 0.01[a]
LA (mm^2)	1.97 ± 0.04	1.85 ± 0.04[a]	1.67 ± 0.04[a]	1.64 ± 0.04[a]
MA/LA (%)	26.97 ± 0.38	29.16 ± 0.73[a]	32.99 ± 0.79[a]	34.13 ± 0.62[a]
SML (layer)	6.17 ± 0.75	7.17 ± 0.73	7.67 ± 0.82[b]	8.17 ± 0.75[b]

[a]$P < 0.05$ compared with the control group.

[b]$P < 0.01$ compared with the control group.

AGEs: Advanced glycation end products; MT: Media thickness; LD: Lumen diameter; MA: Media area; LA: Lumen area; SML: Smooth muscle layers.
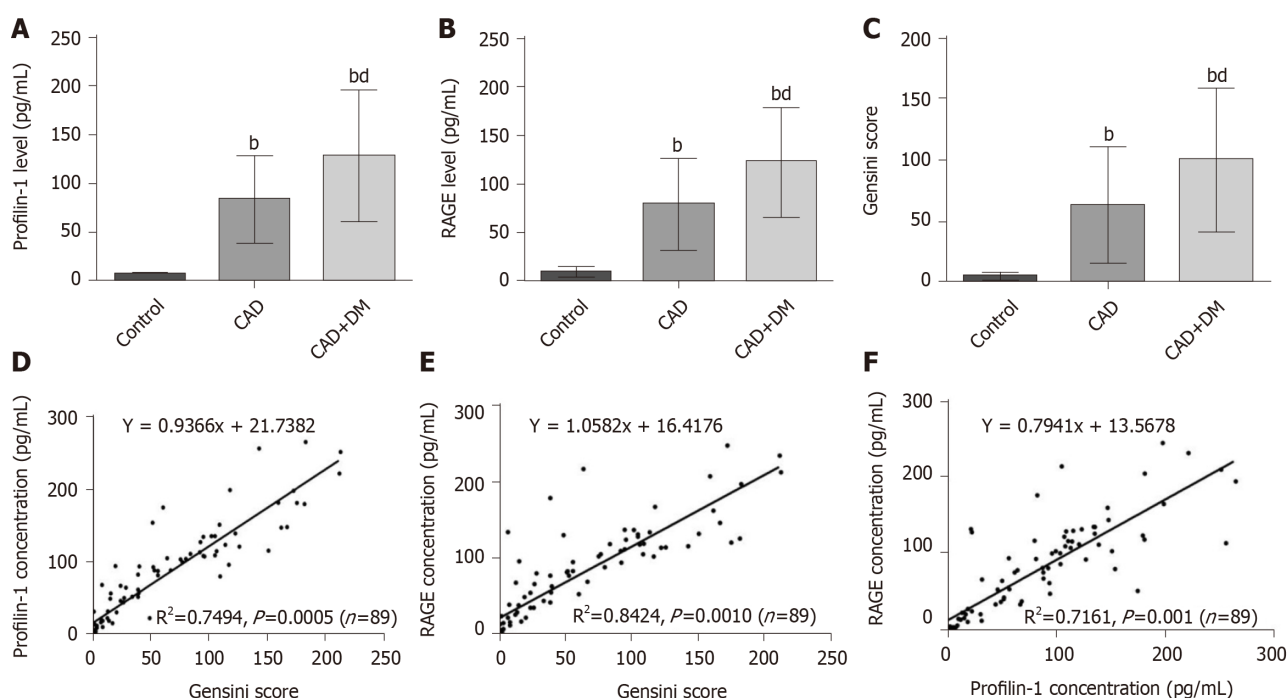
Figure 1 Relationships between profilin-1, receptor for advanced glycation end products, and Gensini scores. A-C: Plasma profilin-1 and receptor for advanced glycation end products (RAGE) levels and Gensini scores in different groups. [b]$P < 0.01$ compared with the control group, [d]$P < 0.01$ compared with the coronary artery disease group; D-F: Pearson correlation analysis showing the relationship between profilin-1 or RAGE levels and Gensini score, as well as the relationship between profilin-1 and RAGE levels. CAD: Coronary artery disease; DM: Diabetes mellitus; RAGE: Receptor for advanced glycation end products.

Expression of profilin-1 mRNA and protein in the thoracic aorta of rats injected with AGEs

Real-time PCR and Western blot analysis of mRNA and protein extracts from thoracic aortae were performed, respectively, to confirm the effect of AGEs on profilin-1 expression *in vivo*. Consistent with the results from our *in vitro* study, the profilin-1 mRNA and protein were expressed at higher levels in the thoracic aorta wall of the AGEs-injection group compared to those of the control group ($P < 0.05$ or $P < 0.01$) (Figure 3A-C). Interestingly, the results of IHC staining showed that the positively stained cells were mainly located in the intima, along with a certain amount in the media, suggesting that profilin-1 was mainly expressed in endothelial cells and SMCs in local aortic AS plaques. Additionally, the expression of profilin-1 in the thoracic aorta of AGEs-injected rats was significantly higher than that of normal rats (Figure 3D). The average optical density, expression-positive area, and integrated
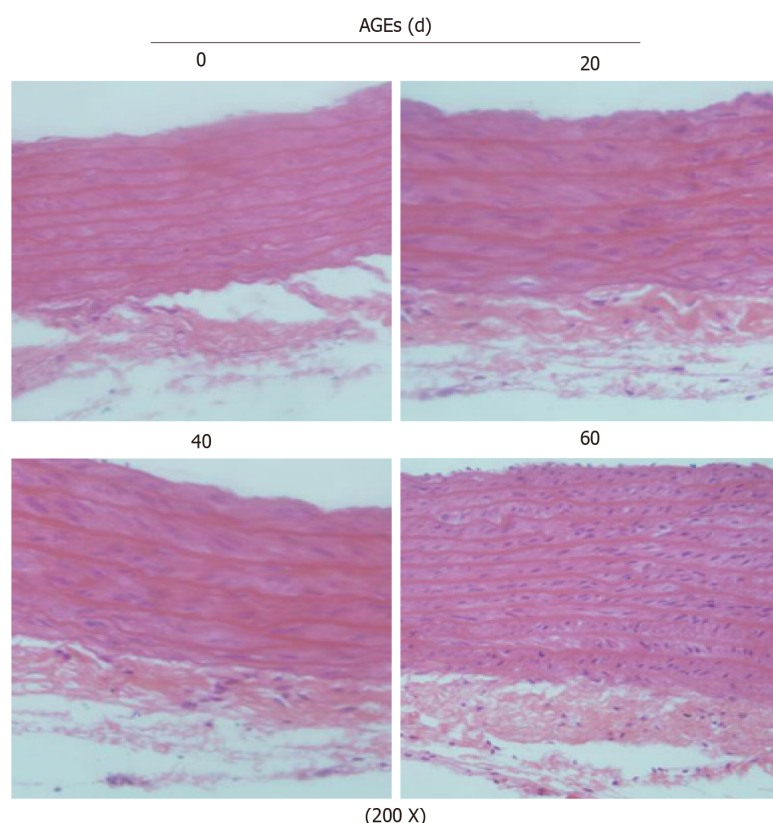
Figure 2 Hematoxylin and eosin staining (200 ×) showing the morphological characteristics of the thoracic aorta in advanced glycation end products-injected rats (*n* = 3). AGEs: Advanced glycation end products.

optical density of profilin-1 in the thoracic aorta were also significantly increased in AGEs-injected rats compared with the control group ($P < 0.001$) (Table 3).

Effect of profilin-1 knockdown on AGEs-induced changes in inflammatory mediators and vascular remodeling

AGEs evoke inflammation in various cell types and organs, including endothelial cells, blood vessels, and the heart[27]. However, the role of AGEs-induced inflammation in vascular remodeling remains unclear. Here, after a chronic tail vein injection of AGEs, the serum levels of ADMA, ICAM-1, and PIIINP were significantly increased compared with the control group (Figure 4B-D) ($P < 0.05$); these changes were accompanied by increased expression of the profilin-1 protein and vascular remodeling in the thoracic aorta (Figure 4E).

We developed a profilin-1-specific shRNA to confirm the role of profilin-1 in the AGEs-mediated production of inflammatory mediators, and the transfection efficiency was confirmed by detecting the expression of the profilin-1 protein. As shown in Figure 4A and E, the profilin-1 shRNA successfully reduced the profilin-1 protein levels in thoracic aortae from AGEs-injected rats. Importantly, silencing of profilin-1 gene expression inhibited AGEs-induced ADMA, ICAM-1, and PIIINP release (Figure 4B-D) ($P < 0.05$) and attenuated AGEs-induced vascular remodeling of the thoracic aorta (Figure 4E). In contrast, the negative control shRNA had no such effect.

Effect of AGEs on RASMC proliferation

As mentioned above, vascular remodeling is an important pathological feature of diabetic macroangiopathy, in which endothelial dysfunction and VSMC proliferation plays a crucial role[28]. As the role of AGEs in endothelial cell injury has been proven [23], we focused on the effects of AGEs on VSMC proliferation *in vitro*. As shown in Figure 5A, after treatment with different concentrations of AGEs (0, 50, 100, 150, 200, or 400 μg/mL) for different times (0, 24, 48, or 72 h), the proliferation of RASMCs was significantly increased compared with that of the control group (Figure 5A, $P < 0.05$), and AGEs concentrations ranging from 100-200 μg/mL exerted the most robust effect on RASMC proliferation. EdU staining and flow cytometry produced similar results (Figure 5B-D).

Table 3 Quantitative analysis of profilin-1 protein levels in the thoracic aorta

Index	Control (*n* = 6)	20 d (*n* = 6)	40 d (*n* = 6)	60 d (*n* = 6)
AOD	0.069 ± 0.002	0.074 ± 0.002[b]	0.079 ± 0.001[b]	0.087 ± 0.005[b]
EPA (mm^2)	202.05 ± 10.33	267.27 ± 24.98[b]	442.28 ± 31.90[b]	599.88 ± 38025[b]
IOD (mm^2)	14.00 ± 0.68	19.71 ± 1.85[b]	34.96 ± 2.43[b]	52.19 ± 5.43[b]

[b]$P < 0.01$ compared with the control group.
The level of the profilin-1 protein was determined using Image-Pro plus immunohistochemical image analysis software. AOD: Average optical density; EPA: Expression-positive area; IOD: Integrated optical density.
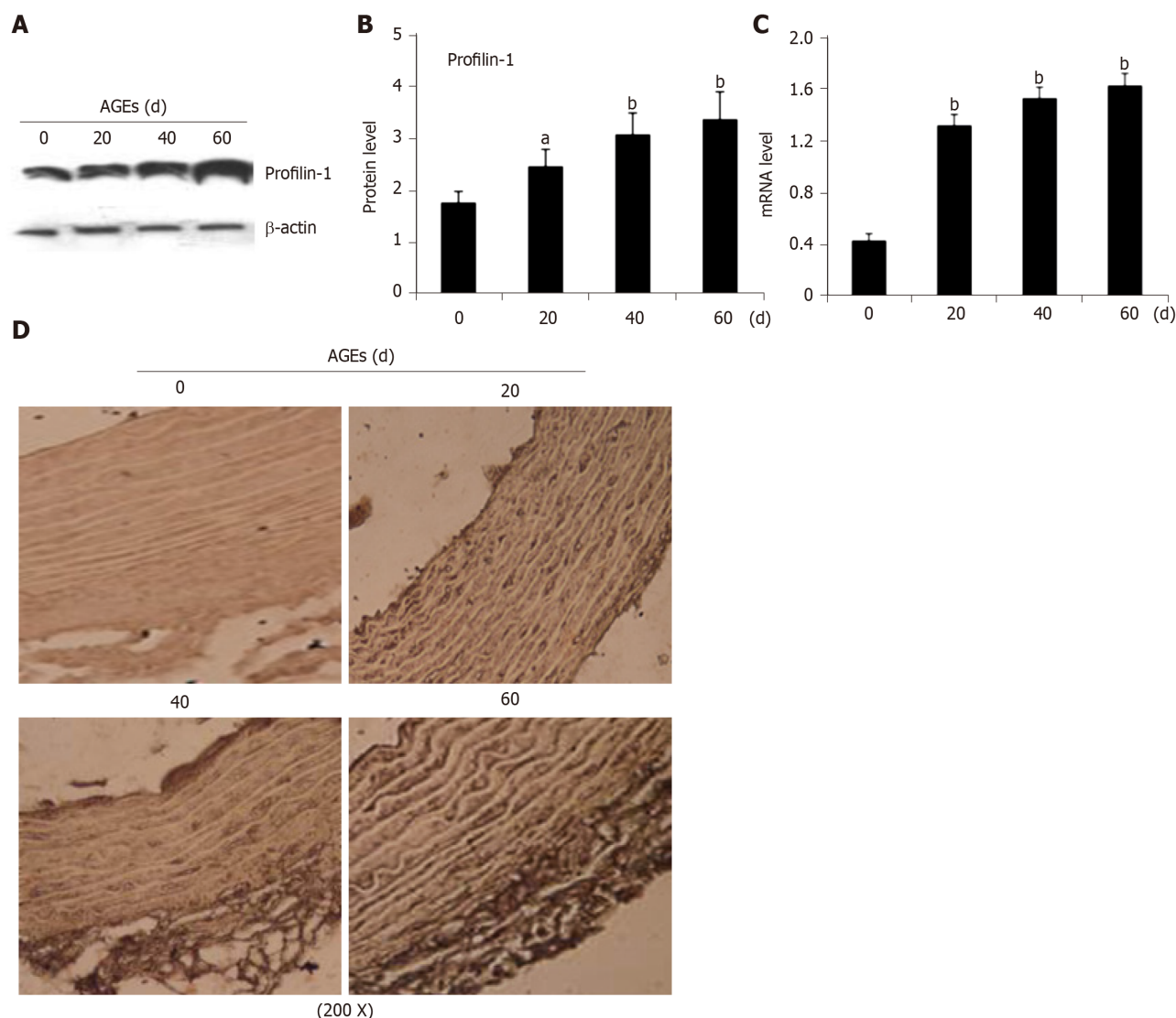
Figure 3 Expression of profilin-1 in the thoracic aortas of advanced glycation end products-injected rats. A-C: Western blot analysis and real-time PCR detection of the expression of the profilin-1 protein and mRNA, respectively, in the thoracic aorta. *n* = 3, [a]$P < 0.05$ compared with the control group, [b]$P < 0.01$ compared with the control group; D: Immunohistochemical staining revealing the expression of the profilin-1 protein in the thoracic aorta. Under a microscope at 200 × magnification, the profilin-1 protein was stained brown using the SABC immunohistochemical method, *n* = 3. AGEs: Advanced glycation end products.

Involvement of the profilin-1 and JAK2/STAT3 pathways in AGEs-induced cell proliferation

Previous studies have shown that the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway mediates the migration and proliferation of VSMCs and that AGEs induce apoptosis and inflammation by activating the JAK2/STAT3 pathway[29,30]. The expression of profilin-1, JAK2, and STAT3 in RASMCs exposed to medium alone or AGEs was examined to explore whether
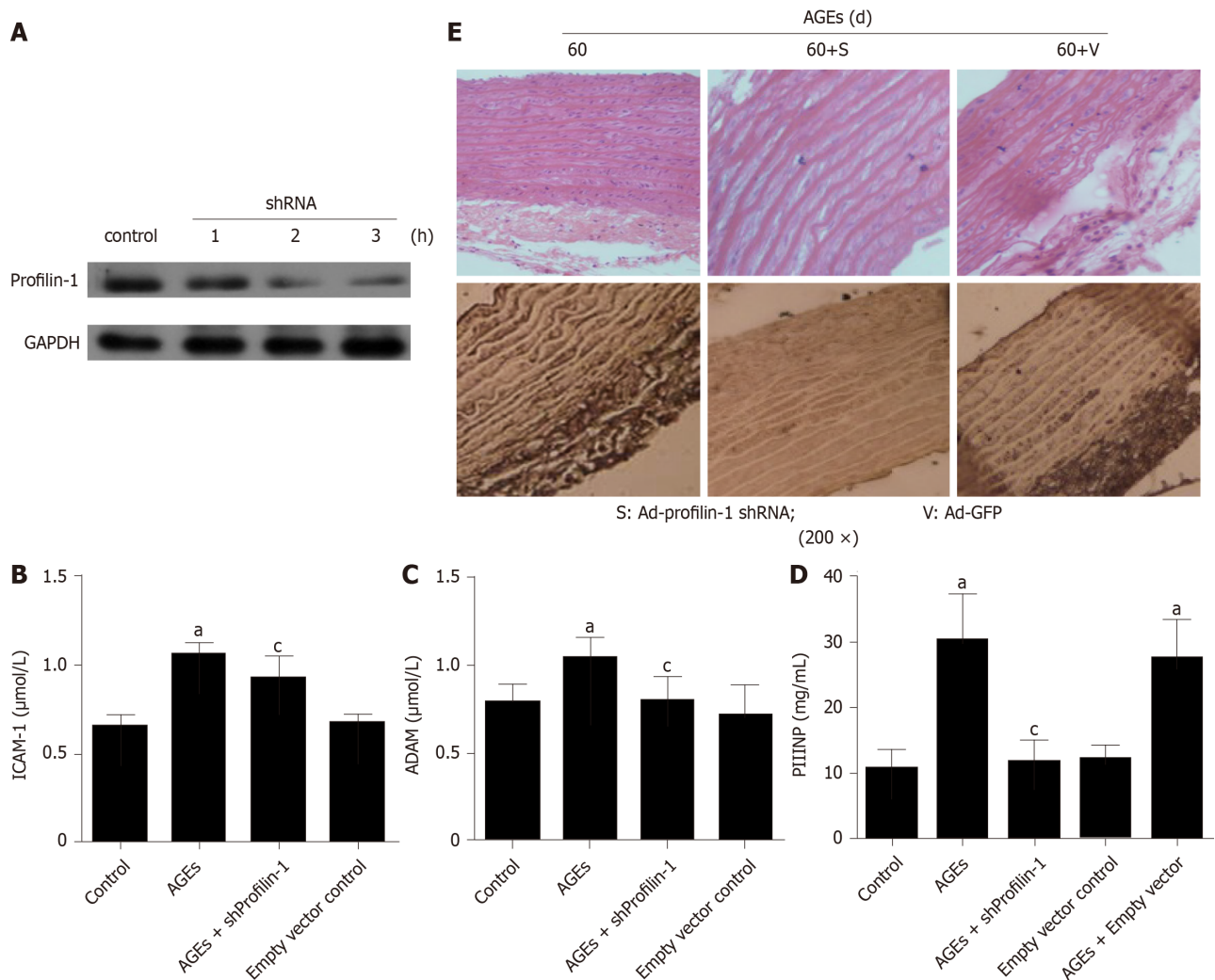
Figure 4 Effect of profilin-1 shRNA on the levels of inflammatory mediators and vascular remodeling in advanced glycation end products-injected rats. A: Western blot analysis of the transfection efficiency of the profilin-1 shRNA, *n* = 3; B-D: ELISA or HPLC was used to detect the intercellular adhesion molecule-1, N-terminal procollagen III peptide, and asymmetric dimethylarginine levels in different groups. *n* = 3, [a]*P* < 0.05 compared with the control group, [c]*P* < 0.05 compared with the advanced glycation end products (AGEs) group; E: Hematoxylin and eosin or immunohistochemistry staining showing the effect of profilin-1 silencing on the morphological characteristics of the thoracic aorta and the expression of the profilin-1 protein in AGEs-injected rats. Under a microscope at 200 × magnification, the profilin-1 protein was stained brown using the SABC immunohistochemical method, *n* = 3. ICAM-1: Intercellular adhesion molecule-1; PIIINP: N-terminal procollagen III peptide; ADMA: Asymmetric dimethylarginine; AGEs: Advanced glycation end products.

profilin-1 and the JAK2/STAT3 pathway are involved in AGEs-induced RASMC proliferation. Compared with the control group, treatment with AGEs at a dose of 100 or 150 µg/mL significantly upregulated the expression of both the profilin-1 mRNA and protein at different time points (*P* < 0.05, Figure 6A-E), which paralleled the increase in the proliferation of RASMCs induced by AGEs. AGEs (200 µg/mL) significantly upregulated the expression of STAT3 at both the mRNA and protein levels, but only upregulated the expression of the JAK2 protein (*P* < 0.05, Figure 6F and G). In addition, the AGEs-induced expression of the JAK2 and STAT3 proteins was effectively blocked by a JAK2 inhibitor (T3042-1) and/or STAT3 inhibitor (T6308-1) (*P* < 0.05, Figure 6H). Pretreatment with T3042-1 (15 µmol/L) or T6308-1 (5 µmol/L) significantly inhibited AGEs-induced RASMC proliferation, as detected by EdU staining and flow cytometry (*P* < 0.05). However, T3042-1 and T6308-1 alone had no effect on RASMC proliferation (Figure 7).

DISCUSSION

Epidemiological and clinical data suggest that diabetic macroangiopathy is the main cause of death in individuals with DM, and major biochemical pathways are involved in the development of diabetic macroangiopathy[27]. Among them, AGEs accumu-
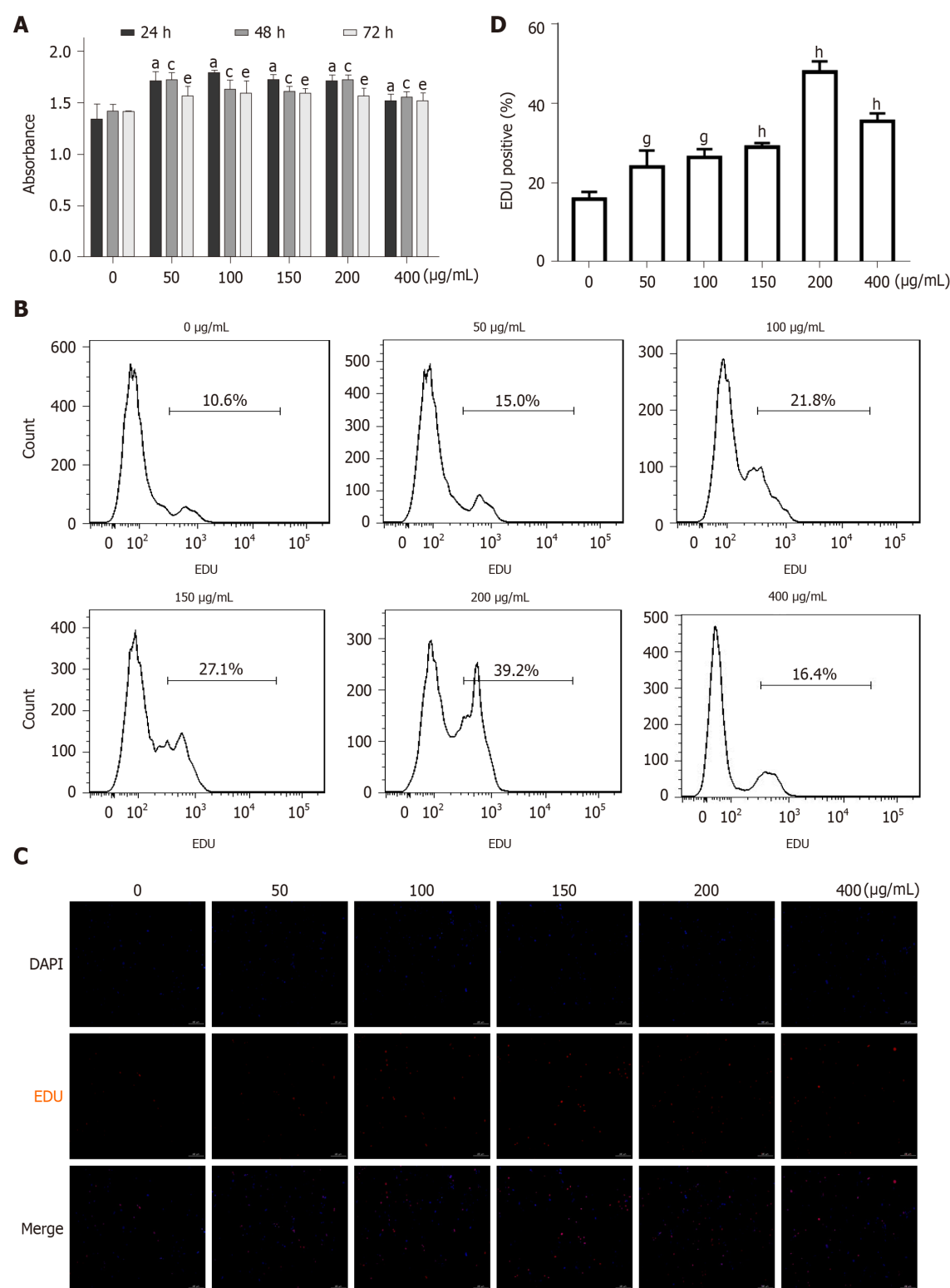
Figure 5 Effect of advanced glycation end products on the proliferation of cultured rat aortic vascular smooth muscle cells (*n* = 6). A: MTT detection of the effect of incubation with advanced glycation end products (AGEs) (0, 50, 100, 150, 200, or 400 μg/mL) for different times (0, 24, 48, or 72 h) on rat aortic vascular smooth muscle cell (RASMC) proliferation. [a]*P* < 0.05 for the comparison of the 24 h control group with the control group, [c]*P* < 0.05 for the comparison of the 48 h control group with the control group, [e]*P* < 0.05 for the comparison of the 72 h control group with the control group; B: Proliferation of RASMCs treated with AGEs (0, 50, 100, 150, 200, or 400 μg/mL) for 24 h quantified using flow cytometry. Data were obtained from six independent experiments (*n* = 6); C: EdU staining showing the effect of incubation with AGEs (0, 50, 100, 150, 200, or 400 μg/mL) for 24 h on RASMC proliferation; D: Proliferation of RASMCs treated with AGEs (0, 50, 100, 150, 200, or 400 μg/mL) for 24 h quantified using fluorescence microscopy. [g]*P* < 0.05 compared with the control group. [h]*P* < 0.01 compared with the control group. EdU: 5-ethynyl-2-deoxyuridine.

lation in tissues and the pivotal role of the AGEs-RAGE signaling pathway are most important for the central pathological features of DM, which rapidly accelerate AS[31, 32]. High serum AGEs levels are associated with an increased incidence of CAD and a
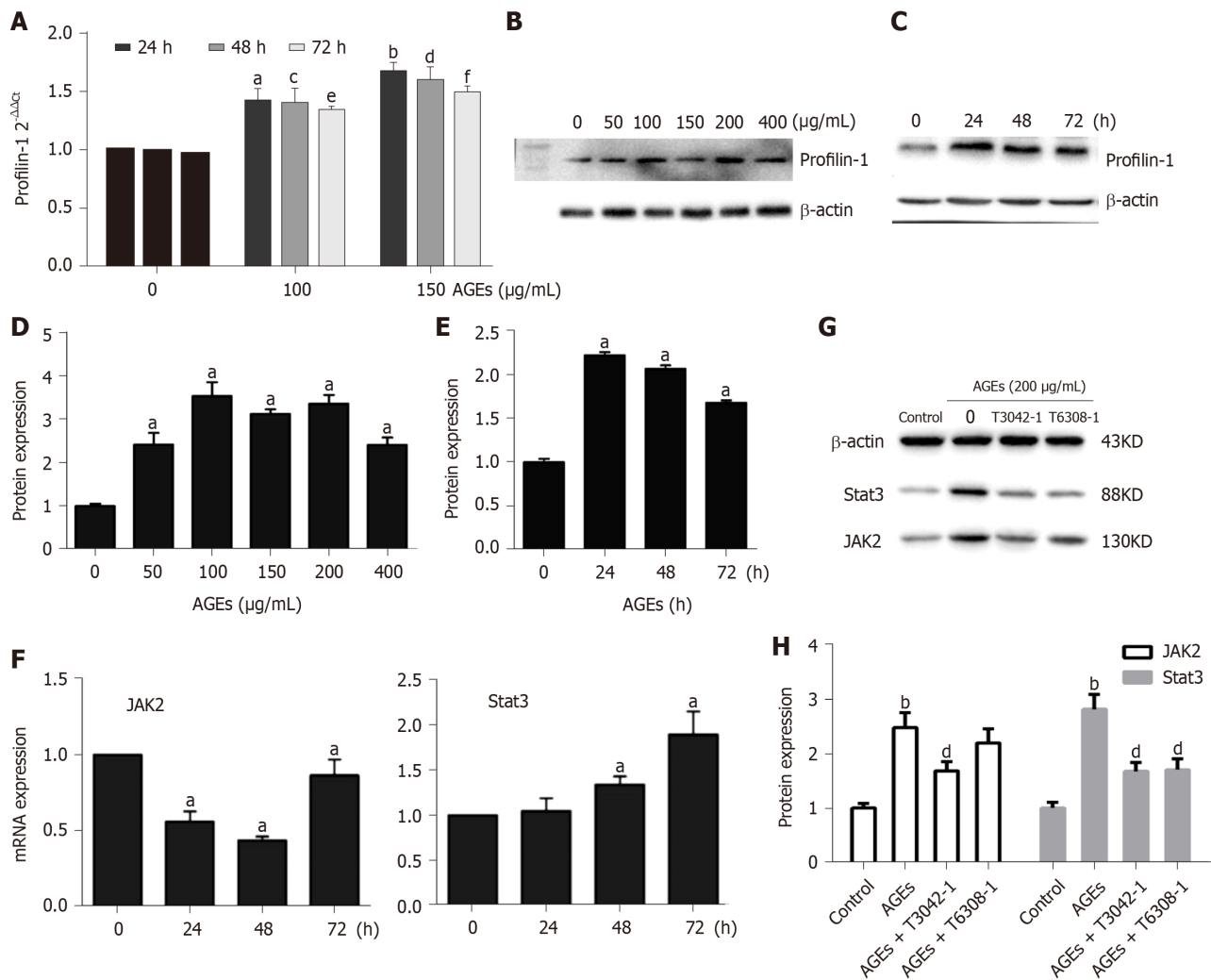
Figure 6 Involvement of profilin-1 and the Janus kinase 2/signal transducer and activator of transcription 3 pathway in advanced glycation end products-induced cell proliferation (*n* = 3). A: RT-PCR analysis of the expression of the profilin-1 mRNA in rat aortic vascular smooth muscle cells (RASMCs) treated with different concentrations of advanced glycation end products (AGEs) (µg/mL) for different times. [a,c,e]*P* < 0.05 compared with the 0 control group, [b,d,f]*P* < 0.01 compared with the 0 control group; B-E: Western blot showing levels of the profilin-1 protein in RASMCs treated with different concentrations of AGEs for different times. [a]*P* < 0.05 compared with the control group; F: RT-PCR analysis of the expression of the Janus kinase 2 (*JAK2*) and signal transducer and activator of transcription 3 (*STAT3*) mRNA in RASMCs treated with 200 µg/mL AGEs for different times. [a]*P* < 0.05 compared with the 0 control group; G-H: Western blot analysis of the effect of 200 µg/mL AGEs on the expression of the JAK2 and STAT3 proteins in RASMCs treated with or without the JAK2 inhibitor (T3042-1) or STAT3 inhibitor (T6308-1). [b]*P* < 0.01 compared with the control group. [d]*P* < 0.01 compared with the AGEs group. AGEs: Advanced glycation end products; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3.

worse prognosis[7]. The present study also found significantly higher levels of RAGE in patients with CAD or CAD + DM than in healthy people, especially in patients with CAD + DM, and the levels of RAGE were positively correlated with Gensini scores. Thus, we focused on the relationship between AGEs and diabetic macroangiopathy characterized by abnormal hyperplasia of vascular smooth muscle, inflammation, and vascular remodeling.

Based on accumulating evidence, the increased proliferation and migration of VSMCs are hallmarks of vascular pathology in individuals with diabetic macroangiopathy[33]. A homogenous population of VSMCs is present within atherosclerotic plaques and responsible for early atherogenesis or preventing fibrous caps from rupturing in advanced plaques[34]. According to recent reports, dietary AGEs in patients with diabetes or an intraperitoneal injection of AGEs in rats causes an impairment in the vascular endothelium[35,36]. In an effort to understand the toxicity of AGEs in vasculopathy *in vivo*, a vascular injury model in rats was established by chronic tail vein injections of AGEs. In the AGEs injection group, thickened aortic walls, looser elastic fibers, broadened endothelial gaps, broken inner elastic tissue, and significant proliferation and disarrangement of VSMCs and luminal stenosis were observed. In addition, the injection of AGEs significantly increased the MT/LD and MA/LA ratios and the thickness of SMLs, which are considered indices of vascular
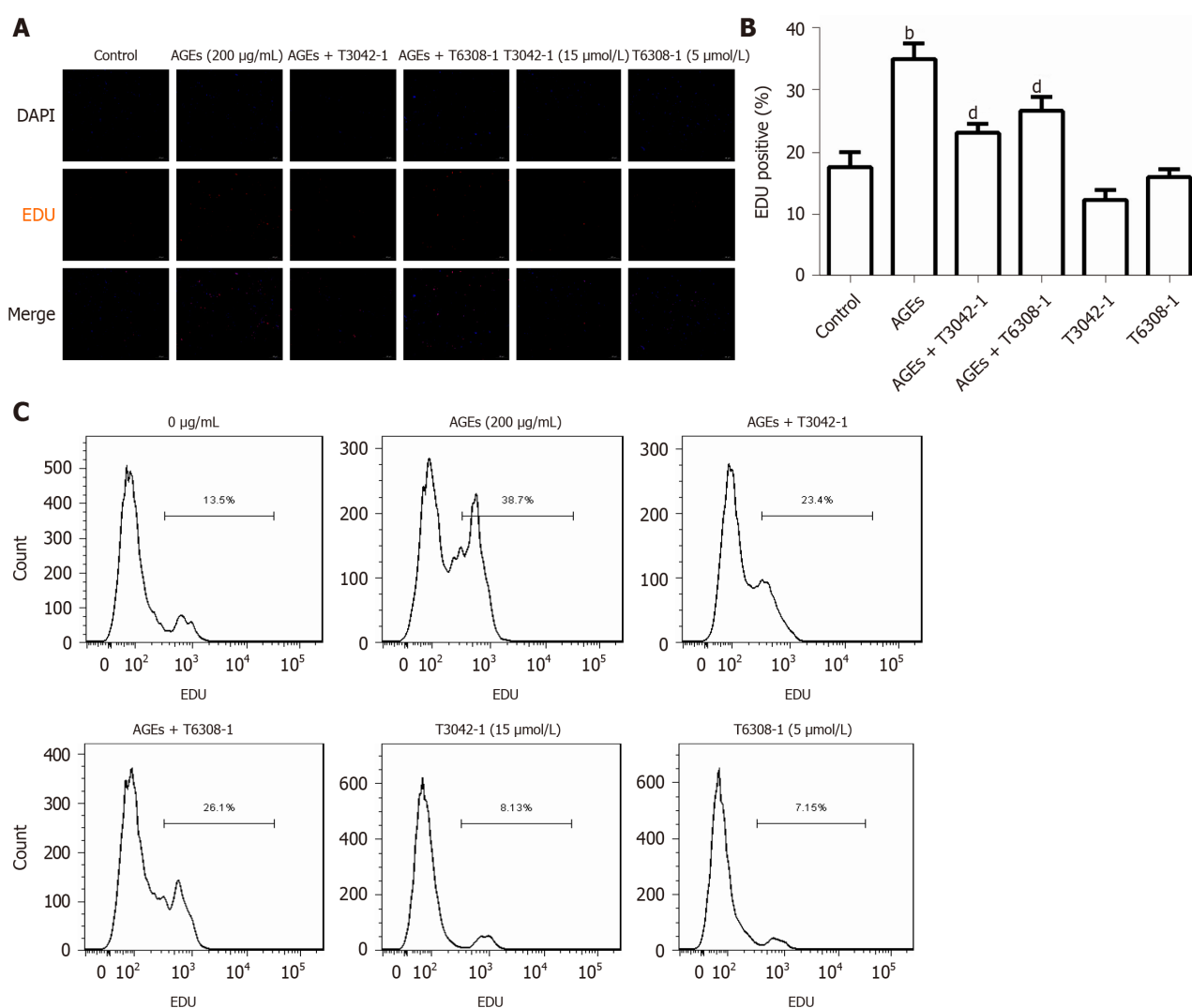
Figure 7 Effects of the Janus kinase 2 inhibitor and signal transducer and activator of transcription 3 inhibitor on advanced glycation end products-induced rat aortic vascular smooth muscle cell proliferation (*n* = 6). Cells were treated with 200 µg/mL advanced glycation end products (AGEs) and/or 15 µmol/L Janus kinase 2 inhibitor (T3042-1) and/or 5 µmol/L signal transducer and activator of transcription 3 inhibitor (T6308-1). A: Fluorescence microscopy image of 5-ethynyl-2-deoxyuridine (EdU) staining; B: Percentage of EDU-positive cells in A. [b]*P* < 0.01 compared with the control group, [d]*P* < 0.01 compared with the advanced glycation end products group; C: Flow cytometry analysis of EdU staining. Data were obtained from six independent experiments. AGEs: Advanced glycation end products; EDU: 5-ethynyl-2-deoxyuridine.

remodeling[37], suggesting that exogenous AGEs directly induce vasculopathy and vascular remodeling *in vivo*. *In vitro*, incubation of VSMCs with AGEs significantly increased the proliferation of VSMCs and activated the proliferative JAK2/STAT3 signaling pathway. JAK2/STAT3 is an important signal transduction pathway that mediates VSMC proliferation and is involved in the process of AS in individuals with diabetes complicated with cardiovascular disease[38]. A previous study reported that AGEs exert proapoptotic and proinflammatory effects on mouse podocytes through the JAK2/STAT3 pathway[28]. Accordingly, we also found that exposure of VSMCs to AGEs induced the activation of JAK2/STAT3 signaling and that both a JAK2 inhibitor and STAT3 inhibitor attenuated the proliferation of VSMCs induced by AGEs. Therefore, AGEs induce VSMC proliferation that subsequently contributes to vascular remodeling under atherosclerotic conditions *via* the activation of the JAK2/STAT3 pathway. However, the precise mechanism of AGEs-induced vascular remodeling deserves further investigation.

Vascular remodeling is proven to be an adaptive process in response to chronic changes in hemodynamic conditions, and increased actin polymerization and stress fiber formation may play pivotal roles in the modulation of cellular morphology and function. Profilin-1, a multifunctional actin-binding protein, plays an essential role in regulating cytoskeletal rearrangement and redistribution by promoting vascular remodeling and inflammation. The most direct evidence is that overexpression of profilin-1 directly induces aortic remodeling in spontaneously hypertensive rats,

manifesting as an increase in vessel size, wall thickness, and collagen content[19,39]. In fact, recent studies have shown that the biological effects of profilin-1 are far greater than those on regulating actin depolymerization and reorganization[40]. Thus, researchers have speculated that profilin-1 is involved in the vascular remodeling induced by AGEs. As shown in our study, the elevated levels of profilin-1 in patients with CAD or CAD + DM were positively correlated with coronary artery stenosis, and AGEs markedly upregulated the expression of profilin-1 in the aorta or cultured VSMCs concomitant with vascular remodeling and the hyperproliferation of VSMCs. Furthermore, profilin-1 silencing also downregulated AGEs-induced profilin-1 expression in aortic tissue and reversed AGEs-induced aortic vascular remodeling, suggesting that profilin-1 may participate in AGEs-induced vascular remodeling.

AS is a chronic inflammatory disorder that is involved in all stages of diabetic macroangiopathy, such as the formation, progression, and rupture of atherosclerotic plaques[41,42]. AGEs/RAGE activation may lead to increased production of proinflammatory and proatherogenic mediators that accelerate diabetes and its complication, AS[30,43]. Recently, the proinflammatory factors ADMA and ICAM-1 have been recognized as early markers of endothelial dysfunction with a high risk of cardiovascular events[44,45]. In patients with myocardial infarction, the levels of PIIINP potentially reflect the extent of extracellular matrix remodeling, and the increase in extracellular matrix contributes to intimal thickening and sclerosis[46,47]. Several studies have reported that AGEs markedly increase ADMA levels in endothelial cells by inducing local ROS production[48,49], and overexpression of profilin-1 upregulates the expression of ICAM-1[23]. In AGEs-injected rats, the production of ADMA, ICAM-1, and PIIINP was markedly increased and aortic vascular remodeling was induced in the present study, changes that were reversed by the inhibition of profilin-1 through shRNA transfection. Based on these results, profilin-1 may participate in AGEs-induced inflammation and vascular remodeling by activating the JAK2/STAT3 pathway, and profilin-1 is expected to become a promising therapeutic target for preventing diabetes-related vascular injury. However, the exact molecular mechanism of profilin-1 in AGEs-induced vasculopathy is worthy of further study.

CONCLUSION

Taken together, our results provide evidence that AGEs induce proatherogenic events such as VSMC proliferation, elevated expression of proatherogenic mediators, and vascular remodeling, which are attenuated by silencing profilin-1 gene expression. These data suggested for the first time that profilin-1 is involved in AGEs-induced aortic atheroma formation, indicating that increased profilin-1 expression in the aorta of patients with diabetes might trigger atherosclerosis-related events. Therefore, drugs targeting profilin-1 may become a potential therapeutic strategy for ameliorating AS secondary to diabetes.

ARTICLE HIGHLIGHTS

Research background
The formation and accumulation of advanced glycation end products (AGEs) contribute to accelerated macrovascular complications, which are the leading cause of morbidity and mortality in patients with diabetes. Profilin-1 plays an important role in vascular remodeling and vascular inflammation.

Research motivation
Currently, few studies have investigated the role of profilin-1 in vasculopathy induced by AGEs, and the mechanism of profilin-1 in diabetic macroangiopathy remains unclear.

Research objectives
The aim of this study was to explore the potential role of profilin-1 in the pathogenesis of atherosclerosis induced by AGEs and to elucidate its probable mechanism.

Research methods

Eighty-nine individuals undergoing coronary angiography were enrolled in the study. Plasma cytokine levels were detected using ELISA kits. Rat aortic vascular smooth muscle cells were incubated with different compounds for different times. Cell proliferation was determined by performing the MTT assay and EdU staining. An AGEs-induced vascular remodeling model was established in rats and histological and immunohistochemical analyses were performed. The mRNA and protein levels were detected using real-time PCR and Western blot analysis, respectively. *In vivo*, shRNA transfection was performed to verify the role of profilin-1 in AGEs-induced proatherogenic mediator release and aortic remodeling. Statistical analyses were performed using SPSS 22.0 software.

Research results

Compared with the control group, plasma levels of profilin-1 and receptor for AGEs (RAGE) were significantly increased in patients with coronary artery disease, especially in those complicated with diabetes mellitus ($P < 0.01$). The levels of profilin-1 were positively correlated with the levels of RAGE ($P < 0.01$); additionally, the levels of both molecules were positively associated with the degree of coronary artery stenosis ($P < 0.01$). *In vivo*, tail vein injections of AGEs induced the release of proatherogenic mediators, such as asymmetric dimethylarginine, intercellular adhesion molecule-1, and the N-terminus of procollagen III peptide, concomitant with apparent aortic morphological changes and significantly upregulated expression of the profilin-1 mRNA and protein in the thoracic aorta ($P < 0.05$ and $P < 0.01$). Downregulation of profilin-1 expression with an shRNA significantly attenuated AGEs-induced proatherogenic mediator release ($P < 0.05$) and aortic remodeling. *In vitro*, incubation of vascular smooth muscle cells (VSMCs) with AGEs significantly promoted cell proliferation and upregulated the expression of the profilin-1 mRNA and protein ($P < 0.05$). AGEs (200 µg/mL, 24 h) significantly upregulated the expression of the signal transducer and activator of transcription 3 (STAT3) mRNA and protein and the Janus kinase 2 (JAK2) protein, which were blocked by a JAK2 inhibitor (T3042-1) and/or STAT3 inhibitor (T6308-1) ($P < 0.05$). In addition, pretreatment with T3042-1 or T6308-1 significantly inhibited AGEs-induced rat aortic vascular smooth muscle cell proliferation ($P < 0.05$).

Research conclusions

The present study proved that AGEs induce proatherogenic events such as VSMC proliferation, proatherogenic mediator expression, and vascular remodeling, which are attenuated by silencing profilin-1 gene expression.

Research perspectives

Profilin-1 is expected to be a promising therapeutic target for the prevention of diabetes mellitus associated with vascular damage. Further studies are needed to develop a targeted drug against profilin-1 and elucidate the exact molecular mechanism of profilin-1 in AGEs-induced vasculopathy.

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ORIGINAL ARTICLE

Basic Study

p66Shc-mediated oxidative stress is involved in gestational diabetes mellitus

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) is associated with a heightened level of oxidative stress, which is characterized by the overproduction of reactive oxygen species (ROS) from mitochondria. Previous studies showed that mitochondrial dysfunction is regulated by dynamin-related protein 1 (Drp1) and p66Shc in GDM.

AIM

The aim was to investigate the expression of Drp1 and p66Shc and their possible mechanisms in the pathogenesis of GDM.

METHODS

A total of 30 pregnant women, 15 with GDM and 15 without GDM, were enrolled. Peripheral blood mononuclear cells and placental tissue were collected. The human JEG3 trophoblast cell line was cultivated in 5.5 mmol/L or 30 mmol/L glucose and transfected with wild-type (wt)-p66Shc and p66Shc siRNA. P66Shc and Drp1 mRNA levels were detected by quantitative real-time polymerase chain reaction. The expression of p66Shc and Drp1 was assayed by immunohisto-

chemistry and western blotting. ROS was assayed by dihydroethidium staining.

RESULTS
The p66Shc mRNA level was increased in the serum ($P < 0.01$) and placentas ($P < 0.01$) of women with GDM, and the expression of Drp1 mRNA and protein were also increased in placentas ($P < 0.05$). In JEG3 cells treated with 30 mmol/L glucose, the mRNA and protein expression of p66Shc and Drp1 were increased at 24 h (both $P < 0.05$), 48 h (both $P < 0.01$) and 72 h (both $P < 0.001$). ROS expression was also increased. High levels of Drp1 and ROS expression were detected in JEG3 cells transfected with wt-p66Shc ($P < 0.01$), and low levels were detected in JEG3 cells transfected with p66Shc siRNA ($P < 0.05$).

CONCLUSION
The upregulated expression of Drp1 and p66shc may contribute to the occurrence and development of GDM. Regulation of the mitochondrial fusion-fission balance could be a novel strategy for GDM treatment.

Key Words: p66Shc; Dynamin-related protein 1; Gestational diabetes mellitus; Oxidative stress; Mitochondrial dysfunction

Core Tip: The role of placental mitochondria in the etiology of gestational diabetes mellitus (GDM) is an emerging area of research. In this study, we report the expression levels of dynamin-related protein 1 (Drp1) and p66Shc in GDM and in the JEG3 human trophoblast cell line. Increased expression of p66Shc was induced by high glucose-activated Drp1 and promoted overproduction of reactive oxygen species, which may be the primary cause of cell damage and apoptosis during the occurrence and development of GDM.

INTRODUCTION

Gestational diabetes mellitus (GDM) is a glucose metabolism disorder that is first discovered or first occurs after pregnancy. It is one of the most common complications of pregnancy, and its incidence is increasing worldwide. Women with a history of GDM have an increased risk of type 2 (T2DM) later in life[1,2]. One study showed that an increased blood glucose level in pregnant women with GDM can lead to an increase in fetal glucose levels, fetal insulin secretion, fetal macrosomia, and a risk of cesarean section and birth trauma, such as vaginal tears, shoulder dystocia, and asphyxia neonatorum[3]. Substantial evidence has shown that the fetuses of women with GDM have an increased risk of developing chronic metabolic diseases, such as obesity, hypertension, cardiovascular disease and T2DM[4].

Currently, the pathogenesis of GDM is still unclear. It is the result of interactions between environmental factors and genetic factors. The pathogenesis of GDM has been proven to be similar to that of DM, which is closely related to inflammatory factors, fat factors, lipids, and so on[1]. The oxidative stress hypothesis is an important advance in the study of placental pathophysiology in recent years. Studies have found that oxidative stress and mitochondrial dysfunction in the placenta are closely related to the onset of GDM[5,6]. Mitochondrial dynamics are necessary for reactive oxygen species (ROS) production[7]. Mitochondria continuously undergo dynamic fusion and fission. Damaged mitochondria are digested by mitochondrial phagocytosis to maintain a healthy mitochondrial pool. Hyperglycemia can lead to fragmentation of mitochondria and the accumulation of damaged mitochondria results in excessive oxidative stress[8]. During the process of metabolism, aerobic cells produce a series of

ROS, including O_2^-, H_2O_2, HO_2^-, ^-OH, *etc*. ROS can activate a series of signal transduction pathways, regulate cell apoptosis and proliferation under different conditions and lead to the onset of gestational diseases[6,9].

p66Shc is a member of the Shc protein family, and an important regulatory protein involved in oxidative stress. It has oxidoreductase activity and regulates mitochondrial oxidative stress, apoptosis, and age-related diseases in mammals. As significant oxidative stress occurs in DM, the relationship between p66Shc and oxidative stress in DM has become a research focus in recent years. Researchers have shown that the expression of p66Shc in peripheral blood mononuclear cells in T2DM patients is significantly higher than that in nondiabetic patients[10], and that high glucose levels enhance the transcription of p66Shc mRNA in cultured human umbilical vein endothelial cells *in vitro*[11]. Whether p66Shc is involved in oxidative stress in the placenta, and the mechanism of mitochondrial dysfunction in GDM have not yet been elucidated. Dynamin-related protein 1 (Drp1) is a dynamic protein that is necessary for mitosis of mitochondria and is primarily located in the cytosol[12]. The equilibrium between fusion and fission is essential for cell integrity and survival. Excessive activation of Drp1 activates complicated mechanisms, disrupting this equilibrium, impairing the function of mitochondria and increasing cell apoptosis[13]. Drp1 has also been proven to contribute to the pathogenesis of obesity, diabetes and cancer[14]. However, the role of Drp1 in the pathogenesis of GDM is still unclear. Here, we investigated the expression of Drp1 and p66Shc in GDM patients and preliminarily discussed the possible mechanism of the pathogenesis of GDM.

MATERIALS AND METHODS

Patients
Fifteen patients diagnosed with GDM and 15 healthy pregnant women were recruited from the Department of Obstetrics in Taian City Central Hospital between May 2016 and February 2017. All women had singleton pregnancies with no other pregnancy complications. The patients with GDM had not received any prenatal treatment and had poor blood glucose control during pregnancy. Women were excluded if they had a history of diabetes mellitus, anemia, or hypertension, a family history of diabetes mellitus, or any other medical complications. The women in the two groups were matched for age, gestational age, number of pregnancies, parity, nutritional status, and educational level. All pregnant women were tested with a standardized oral glucose tolerance test. GDM was diagnosed by one or more of the following criteria: (1) A fasting blood sugar level ≥ 5.1 mmol/L; and (2) A blood sugar level ≥ 10.0 mmol/L 1 h after consuming glucose and ≥ 8.5 mmol/L 2 h after consuming glucose. The study was approved by the Ethics Committee of Taian City Central Hospital and was in accord with the code of ethics of the World Medical Association. All participants were fully informed about the study and provided written informed consent.

Collection and processing of specimens
Blood was collected from elbow veins of fasted pregnant women into anticoagulant tubes with ethylenediaminetetraacetic acid (EDTA, TaKaRa, China) before delivery. Mononuclear cells were extracted over 30 min in 1 mL Total RNA Extraction Reagent (TRIzol, TaKaRa, China), and the cells were homogenized, mixed, and stored at −80 °C. Placental specimens were cut from the maternal surface near the umbilical cord, avoiding obvious fibrosis and calcified areas. One piece was quickly stored in liquid nitrogen, and the other was fully rinsed, fixed in 10% formaldehyde solution for at least 24 h, placed in an embedding box, dehydrated, and embedded in paraffin. Placental tissues (50–100 mg) were homogenized adequately, 1 mL TRIzol was added, and the tissues were incubated for 5 min at room temperature to ensure complete dissociation of the nucleic acid protein complex.

Cell culture
The human trophoblast cell line JEG-3 was purchased from Shanghai Cell Bank (China) and cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 100 ng/mL streptomycin (Sangon Biotech, Shanghai) at 37 °C in a 5% CO_2 incubator. The cells were treated with 5.5 mmol/L or 30 mmol/L glucose and then cultured in a high-glucose environment for 24 h, 48 h or 72 h and collected for further analysis.

Cell transfection

JEG3 cells were incubated in a 6-well plate until the cell density reached 90%–95% on the day of transfection. Plasmid DNA expressing p66Shc was generated by Biosune Biotechnology (Shanghai Co., Ltd., China) using a green fluorescent protein granule-N1 (pEGFP-N1) plasmid, and p66Shc-siRNA (5'-GCAAACAGAUCAUCGCCAATT-3' sense and 5'-UUGGCGAUGAUCUGUUUGCTT-3' antisense) were generated by Shanghai GenePharma (Shanghai, China). Following the manufacturer's instructions, 240 µL serum-free MEM and 10 µL Lipofectamine 2000 were added to each well, mixed, and incubated for 5 min. Then, 4 µg plasmid was diluted in 246 µL serum-free MEM and added to the solution in each well and incubated for 20 min at room temperature before adding 500 µL of the plasmid-Lipofectamine 2000 mixture with gentle mixing and incubation at 37 °C in a CO_2 incubator. After 4–6 h, the medium was replaced with complete medium containing FBS. The transfection efficiency was determined 48 h later.

RNA isolation

Homogenates of blood mononuclear cells and of placenta tissue were treated with 0.2 mL chloroform, mixed for 15 s and incubated for 5 min at room temperature. Then, the specimens were centrifuged at 12000 × *g* for 15 min. The supernatant was transferred to a new tube, treated with 0.5 mL 100% isopropanol, mixed, incubated for 10 min, and then centrifuged at 12000 × *g* for 10 min. The RNA precipitate at the bottom of the tube was then washed with a mixture of TRIzol and absolute ethanol, dried, and resuspended in 0.1% diethyl pyrocarbonate solution. The RNA concentration was then measured with an ultraviolet spectrophotometer (Amersham Biosciences, United States).

Quantitative real-time (qRT)–PCR

cDNA was isolated from all of the samples by reverse transcription in a 10 µL system according to the instructions of the PrimeScript™ RT Reagent Kit (Takara, China). The primer sequences are listed in Table 1. qRT-PCR was carried out in a MicroAmp® Optical 96-well reaction plate using a qRT-PCR instrument (ABI, United States). The reaction volume was 10 µL, and the reaction system included 0.8 µL primers, 1 µL diluted cDNA template, 3.2 µL enzyme-free water, and 5 µL SYBR Green (Takara, China). Amplification was performed in quadruplicate using the following cycling parameters: 95 °C for 30 s; 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s; and melting curve analysis (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s). β-Actin was used as an internal standard. The relative levels of target mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method.

Hematoxylin-eosin staining and immunohistochemistry

Placental tissue was sectioned at 6 µm on a tissue microtome, heated for 30 min at 65 °C, dewaxed and hydrated. The sections were soaked in hematoxylin-eosin solution for 5 min, washed and then placed in 1% hydrochloric acid alcohol for 2 s and ammonia for 10 s. After that, the sections were stained with 1% eosin solution for 10 min, washed, dehydrated step by step, sealed, and imaged.

For immunohistochemistry, the sections underwent heat-induced antigen retrieval for 15 min and cooled to room temperature. Endogenous peroxides were inactivated by incubation with 0.3% H_2O_2 for 15 min, the sections were washed three times with 1× PBS, and incubated with sheep serum for 30 min at 37 °C. After that, the sections were incubated with primary antibodies overnight at 4 °C. The next morning, the sections were rewarmed for 30 min at 37 °C, washed, and incubated with biotin-labeled goat anti-rabbit IgG secondary antibody solution for 20 min at 37 °C. Then, the sections were incubated with horseradish peroxidase (HRP) enzyme-labeled streptomyces ovalbumin working solution for 15 min and washed three times with 1× PBS. Diaminobenzidine chromogen solution (ZSGB-BIO, China) and Harris' hematoxylin (Solarbio, China) were then added to the sections in sequence for a few minutes. Finally, the sections were dehydrated, mounted and sealed. Photographs were taken with a microscope (Olympus, Japan).

Reactive oxygen species detection by dihydroethidium

Placental sections were dewaxed, hydrated, and soaked for 10 min in 1× PBS; cell culture plates were washed two times with 1× PBS. The sections and cell plates were then incubated with diluted dihydroethidium (DHE) solution (Beyotime, China) for 30 min at 37 °C in the dark, washed, and sealed with glycerin. Images were taken using a fluorescence microscope (Olympus, Japan).

Table 1 Primer sequences

Primers	Forward	Reverse
p66Shc	5'-GCCAAAGACCCTGTGAATCAG-3'	5'-GTATTGTTTGAAGCGCAACTCG-3'
Drp1	5'-TGCCGTGAACCTGCTAGATG-3'	5'-GCCTTTGGCACACTGTCT TG-3
β-actin	5'-CTCACCATGGATGATGATATCGC-3'	5'-AGG AATCCTTCTGACCCATGC-3'

Drp1: Dynamin-related protein 1.

Western blotting

After washing JEG3 cells two times with 1× PBS, total protein was extracted using a protein extraction reagent (Beyotime, China) containing protease and phosphatase inhibitors. A bichinchonic acid (BCA) protein assay it (Beyotime, China) was used to measure the protein concentration with a NanoDrop 2000c ultramicro spectropho-tometer (Thermo Scientific, USA). After 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels were prepared, the extracted proteins and ladders were added to the wells, electrophoresed, and transferred onto a nitrocellulose filter membrane (Pall, United States). The membrane was then incubated in 5% skim milk overnight at 4 °C and washed three times with 1× PBST. Immunoblotting was performed using anti-p66Shc (1:500, Abcam, UK), anti-Drp1(1:100, Abcam, UK), rabbit polyclonal anti-GAPDH IgG (1:500, CST, USA) and mouse anti-β-actin IgG (1:1000, CST, United States) antibodies at room temperature for 2 h. After washing three times with 1× PBS, the polyvinylidene difluoride (PVDF) membrane was incubated with an HRP-conjugated rabbit anti-mouse IgG secondary antibody (1:5000, Zsbio, China) for 1 h and then washed. The specific bands were detected using Pierce™ enhanced chemolumin-escence (ECL) western blotting substrate (Millipore, USA) with a Bio–Rad electro-phoresis image analyzer (Bio–Rad, United States). The gray values were analyzed with image analysis software.

Statistical analysis

SPSS Statistics 24.0 (IBM, United States) and GraphPad Prism 5.0 (GraphPad Software, United States) were used for statistical analysis. The clinical characteristics and biochemical indices were compared by *t*-tests or chi-squared tests. A P value < 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics

Fifteen GDM patients and 15 women with healthy pregnancies were included in this study (Table 2). The mean maternal age and gestational age were not significantly different between the two groups (P > 0.05). The prepregnancy body mass index (BMI) and late pregnancy BMI of the GDM group were significantly higher than those of the control group (both P < 0.001). The fasting blood glucose level was significantly higher in the GDM group than in the control group (P < 0.001).

Higher levels of p66Shc, Drp1 and ROS in GDM patients

To investigate the roles of p66Shc and Drp1 in GDM, we measured the p66Shc mRNA level in the maternal serum and the expression of p66Shc and Drp1 mRNA in the placentas of GDM patients and in patients with healthy pregnancies by qRT-PCR. The protein expression of p66Shc and Drp1 was determined by immunohistochemical staining, and the level of ROS in the placentas was determined by DHE staining.

The results revealed significantly higher p66Shc mRNA levels in the maternal serum of GDM patients than in control group patients (P < 0.01) (Figure 1A). The mRNA expression levels of p66Shc (P < 0.01) and Drp1 (P < 0.05) were also significantly increased in the placentas of GDM patients compared with those of patients with normal pregnancies (Figure 1B). HE staining showed thickened arterioles of placental villi, narrowed lumens, poorly matured terminal capillaries and fewer terminal capillaries in the placentas of GDM patients. The levels of ROS were also significantly increased in the placentas of GDM patients (Figure 2A). Immunohisto-chemical staining showed that the expression levels of p66Shc and Drp1 were

Table 2 Clinical characteristics of the study patients

Characteristic	Control group, *n* = 15	GDM group, *n* = 15
Maternal age in yr	31.8 ± 0.77	32.5 ± 1.02
Gestational age in wk Pre-pregnant BMI in kg/m²	38.5 ± 0.4221.11 ± 0.40	38.3 ± 0.3524. 46 ± 0.51[a]
Late pregnant BMI in kg/m²	25.29 ± 0.43	30.63 ± 0.53[a]
FPG in mmol/L	4.17 ± 0.09	5.88 ± 0.15[a]

[a]$P < 0.001$ *vs* control.

BMI: Body mass index; FPG: Fasting plasma glucose; GDM: Gestational diabetes mellitus.
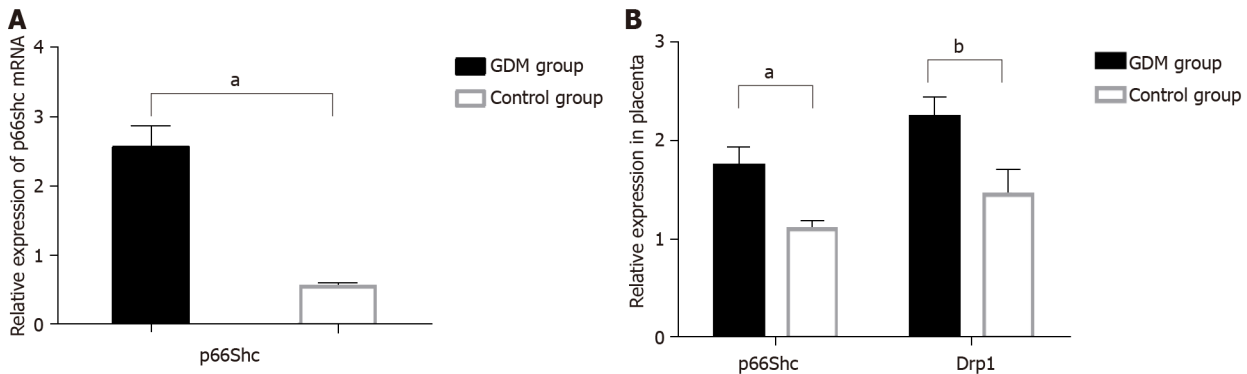

Figure 1 Expression of p66shc mRNA in peripheral blood mononuclear cells and placental tissue in women with and without gestational diabetes mellitus. A: p66Shc mRNA expression in peripheral blood mononuclear cells determined by quantitative real-time PCR; B: Expression of p66Shc and dynamin-related protein 1 mRNA in placentas determined by qRT-PCR. [a]$P < 0.01$ *vs* control; [b]$P < 0.05$ *vs* control. Drp1: Dynamin-related protein 1'; GDM: gestational diabetes mellitus.

significantly higher in the placentas of the GDM group than in the placentas of the control group (Figure 2B).

Hyperglycemia promotes the expression of p66Shc and Drp1 in JEG3 cells

To further explore the roles of p66Shc and Drp1 in GDM, we measured the expression of p66Shc and Drp1 in JEG3 cells treated with different concentrations of glucose by qRT-PCR and Western blotting. The results showed that the mRNA level and protein expression of Drp1 and p66Shc were significantly increased (both $P < 0.05$) in the 30 mmol/L group compared with the 5.5 mmol/L group at 48 h (Figure 3A and B) and were also significantly increased at 0 h and 24 h (both $P < 0.05$), at 48 h (both $P < 0.01$), and at 72 h ($P < 0.01$ and $P < 0.001$) in the 30 mmol/L group. The longer the treatment time, the more significant the increase was (Figure 3C and D). Moreover, ROS levels were significantly elevated in the 30 mmol/L group compared with the 5.5 mmol/L group at 24 h (Figure 3E).

p66Shc upregulates the expression of Drp1 and the level of ROS

To determine the possible relationship among p66Shc, Drp1, and ROS, we altered the expression of Drp1 by transfecting cells with wt-p66Shc and p66Shc siRNA. The results showed that the expression of Drp1 was significantly upregulated in JEG3 cells after transfection with wt-p66Shc ($P < 0.01$) but significantly downregulated after transfection with p66Shc siRNA ($P < 0.05$, Figure 4A and B). The levels of ROS were also significantly increased in JEG3 cells after transfection with wt-p66Shc and significantly decreased after transfection with p66Shc siRNA (Figure 4C).

DISCUSSION

Oxidative stress refers to an imbalance between oxidation and antioxidation *in vivo*. Excess ROS and reactive nitrogen species damage tissues and cell structure and function through oxidation of proteins, lipids, DNA and other biological macro-
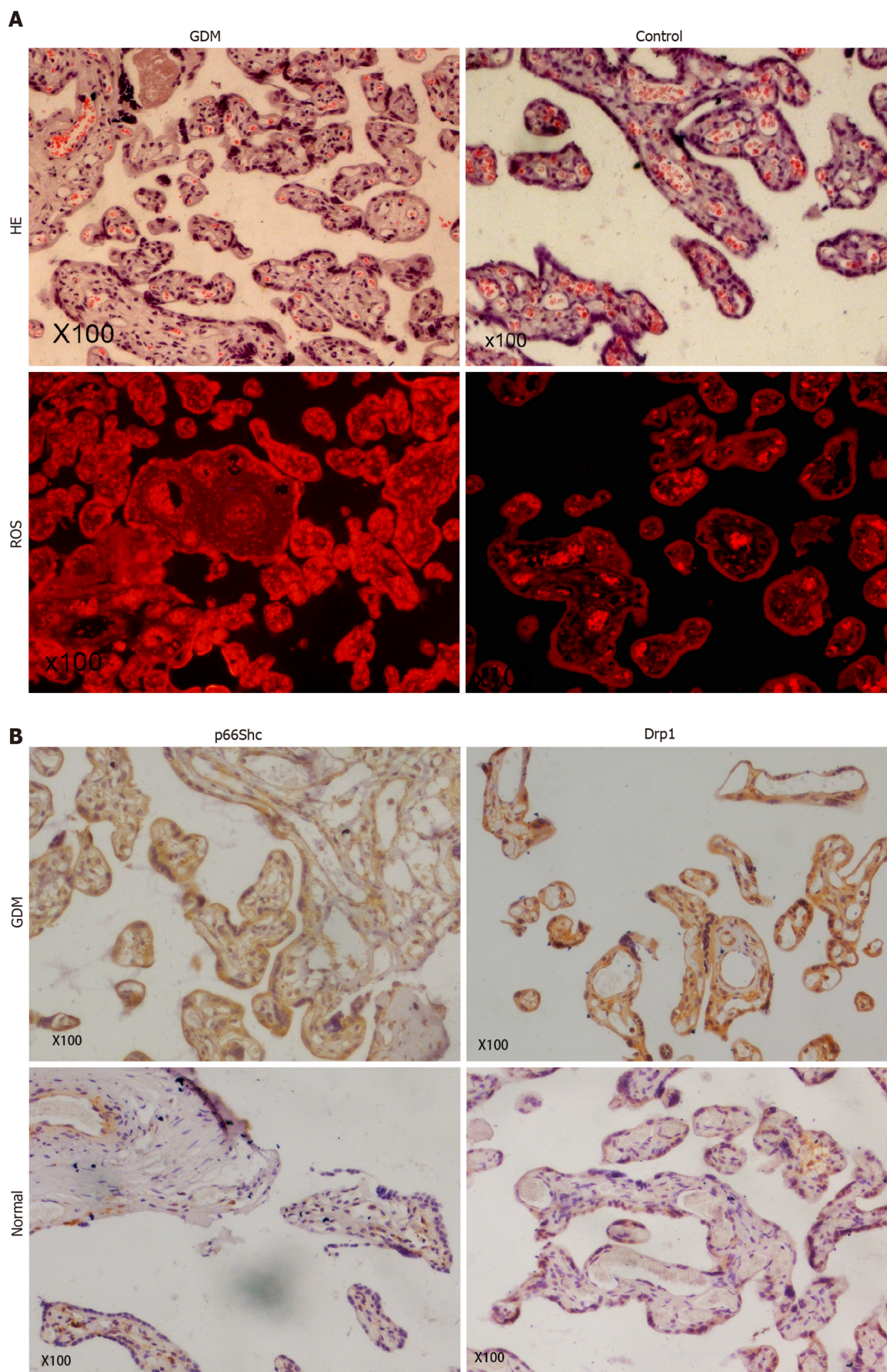
Figure 2 Pathophysiological changes in placentas from women with and without gestational diabetes mellitus. A: Pathological changes in the placenta were observed by hematoxylin-eosin staining (× 100). The level of reactive oxygen species in the placentas was determined by dihydroethidium staining (× 100); B: Protein expression of p66Shc and dynamin-related protein 1 in placentas were determined by immunohistochemical staining (× 100). Drp1: Dynamin-related

molecules[15,16]. In DM patients, hyperglycemia, hyperlipidemia, and inflammation stimulate different tissues to overproduce ROS to levels that exceed the capacity of antioxidant enzymes. The accumulation of these active molecules directly causes chronic oxidative stress by oxidizing and damaging protein, lipid, and DNA molecules that are involved in insulin resistance (IR) and results in the functional decline in the number of pancreatic β-cells in patients with hyperglycemia and hyperlipidemia[8]. Oxidative stress is also observed in patients with GDM, and their antioxidant capacity is significantly decreased [5,17]. Excess ROS can further lead to hypertension, IR and hyperglycemia[9,18]. In our study, we found that the levels of ROS in the placentas of GDM patients were significantly higher than those in the placentas of healthy pregnant women. The levels of ROS were also significantly higher in JEG3 cells treated with high glucose than in those treated with low glucose, which indicated that ROS levels were higher in GDM.

Physiological oxidative stress is indeed a protective and adaptive mechanism of the body during normal pregnancies. However, excessive active oxygen can disrupt the balance between oxidation and antioxidation, cause pathological oxidative stress, and ultimately lead to tissue damage in GDM[6,19]. A previous study showed that placental cell biology and mitochondrial dysfunction are central to the pathophysiology of many gestational diseases, such as GDM, preeclampsia, preterm birth, and fetal growth restriction[20]. Mitochondria are organelles that have an important role in cell growth, metabolism, apoptosis, and aging in eukaryotic cells, and are important drug targets for many prevalent diseases[21]. It was found that increased mitochondrial mass and mitochondrial (mt)DNA content are early molecular events in response to oxidative stress in human cells[12]. Mitochondrial dysfunction can lead to IR, T2DM, obesity and other metabolic diseases[13,22]. It has been shown that the production of excess ROS in DM patients can lead to opening of mitochondrial permeability transition pores and mitochondrial swelling, and induce activation of cell apoptosis and the release of certain substances into the maternal circulation, which further impairs the function of pancreatic β-cells and causes the maternal symptoms of GDM[23,24].

In a pathological pregnancy, the morphology and content of the mitochondria in the placenta change adaptively with changes in ROS levels, and the changes are accompanied by simultaneous damage and adaptive regulation[25]. Mitochondrial morphology and density are regulated by a reduction in mitochondrial fusion and an increase in mitosis[26]. In obese women with GDM, there is no significant change in the biogenesis of placental mitochondria, but a dynamic change has been found in placental mitochondrial morphology[27]. The mitochondrial changes contribute to ROS overproduction under hyperglycemic conditions and may become a target to control the production of ROS in hyperglycemia-associated disorders[28].

Mitochondrial dynamics are regulated by mitotic/fusion proteins, such as Drp1. Overexpression of Drp1 accelerates mitochondrial fission and promote the production of mitochondrial fragmentation and ROS[10]. Drp1 is activated in high glucose-treated cardiovascular cells, participates in fission-mediated fragmentation of mitochondrial tubules, enhances the production of mitochondrial ROS, opens the membrane permeability transition pore, and ultimately leads to cell injury and apoptosis[7]. In this study, high expression of Drp1 was found in the placentas of GDM patients. In addition, Drp1 was more highly expressed in a high-glucose environment than in a low-glucose environment, and its expression level increased with time under high-glucose conditions. The result shows that abnormal mitochondrial function is dominated by Drp1 at the onset of GDM. Notably, ROS levels were increased in the placentas of GDM patients and JEG3 cells treated with high glucose. Therefore, we speculated that Drp1 overexpression resulted in abnormal mitochondrial morphology and function and excessive production of ROS, promoting IR and human trophoblast cell apoptosis and contributing to the pathogenesis of GDM. Abbade *et al*[29] showed that placental mitochondrial dynamics are skewed toward fusion in GDM, as demonstrated by transmission electron microscopy and changes in the expression of key mechanochemical enzymes such as OPA1 and active phosphorylated Drp1. They found decreased Drp1 levels in placental tissue from women with GDM, which is contrary to our results. However, he also mentioned that proper glycemic control an important factor in GDM patients. In our study, we selected untreated GDM patients with poor glycemic control during pregnancy. However, the limited sample size hindered the capacity to assess the impact of Drp1 on the placentas of women with
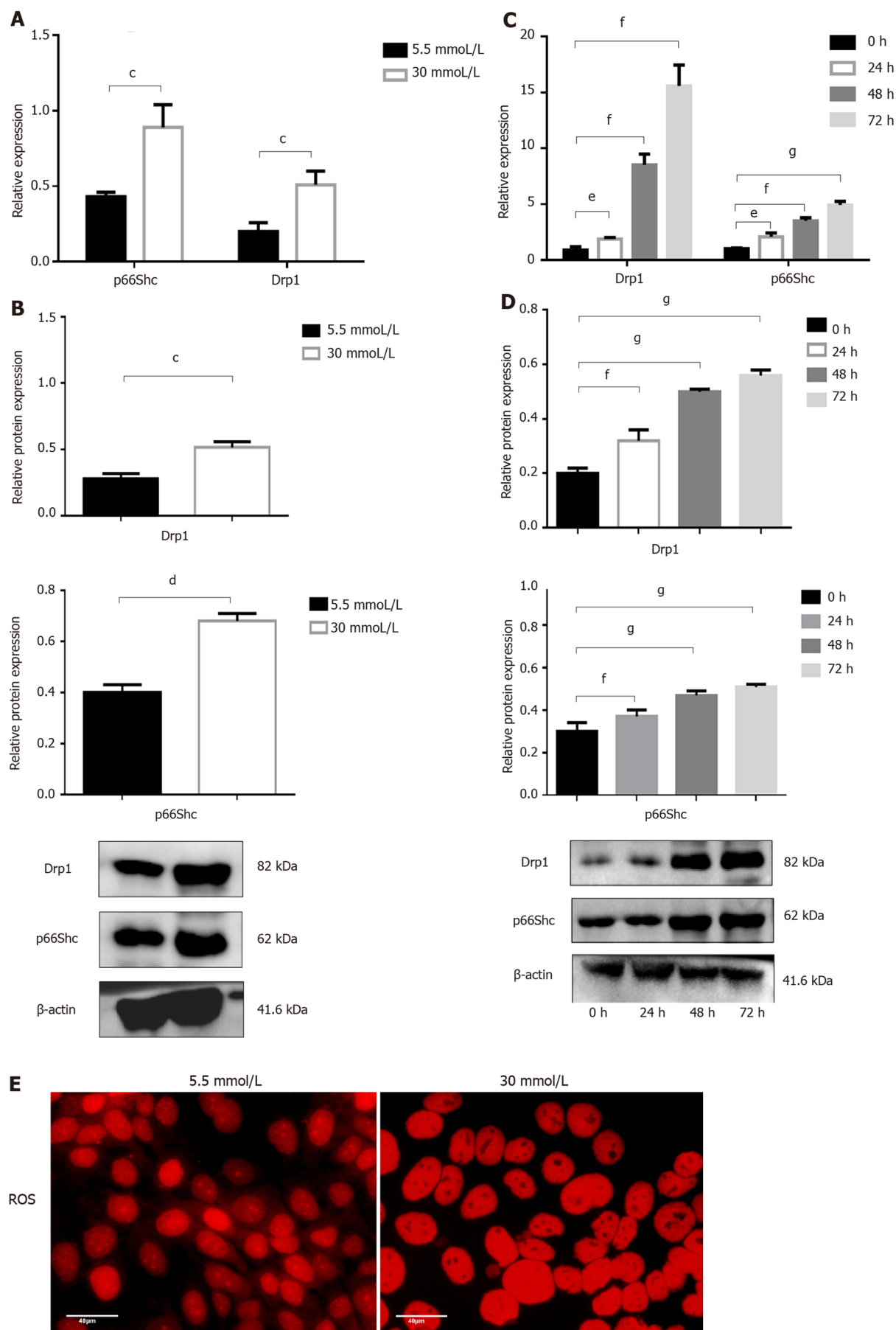
Figure 3 Expression of p66Shc and dynamin-related protein 1 in JEG3 cells treated with 5.5 mmol/L or 30 mmol/L glucose. A: mRNA expression of dynamin-related protein 1 (Drp1) and p66Shc at 24 h were determined by quantitative real-time PCR); B: Protein expression of Drp1 and p66Shc at 24

h was determined by western blotting; C: Drp1 and p66Shc expression of at 0 h, 24 h, 48 h, and 72 h with 30 mmol/L was determined by quantitative real-time PCR; D: Expression of Drp1 and p66Shc at 0 h, 24 h, 48 h, and 72 h with 30 mmol/L were determined by western blotting; E: Reactive oxygen species at 24 h was determined by diluted dihydroethidium staining. $^{c}P < 0.05$ *vs* 24 h in 5.5 mmol/L; $^{d}P < 0.01$ *vs* 24 h in 5.5 mmol/L; $^{e}P < 0.05$ *vs* 0 h in 30 mmol/L; $^{f}P < 0.01$ *vs* 0 h in 30 mmol/L; $^{g}P < 0.001$ *vs* 0 h in 30 mmol/L. Drp1: Dynamin-related protein 1; ROS: reactive oxygen species.
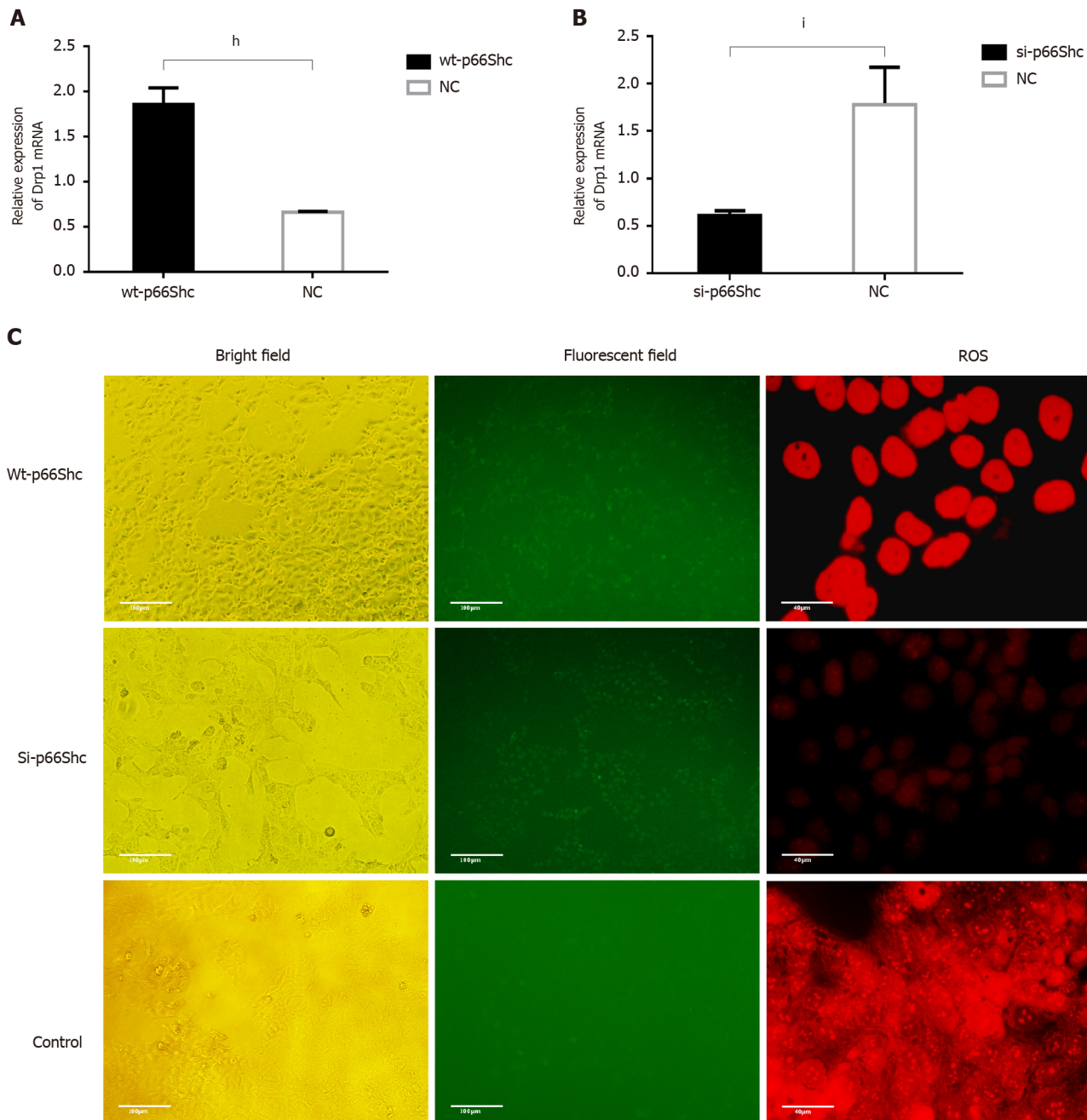

Figure 4 Expression of dynamin-related protein 1 and reactive oxygen species in JEG3 cells after transfection with wt-p66Shc, si-p66Shc, and negative controls. A: mRNA expression of dynamin-related protein 1 (Drp1) in the wt-p66Shc and negative control (NC) cells was determined by quantitative real-time PCR; B: The mRNA expression of Drp1 in the si-p66Shc and NC groups was determined by qRT-PCR; C: The levels of ROS in the wt-p66Shc, si-p66Shc and NC groups were determined by diluted dihydroethidium staining. $^{h}P < 0.01$ *vs* NC; $^{i}P < 0.05$ *vs* NC. wt-p66Shc: Transfected with plasmid DNA; si-p66Shc: Transfected with siRNA; NC: Negative control; ROS: Reactive oxygen species; Drp1: Dynamin-related protein 1.

GDM.

p66Shc is an important regulatory protein of oxidative stress in the mitochondrial pathway. After phosphorylation at Ser36, p66Shc is transported to the mitochondrial membrane and generates mitochondrial ROS through the oxidation of cytochrome c [30]. In a DM mouse model, the expression of p66Shc was found to be significantly increased. However, knockout of the p66Shc gene reduced the generation of oxidative

stress in renal tissues and delayed the progression of disease, suggesting that p66Shc is involved in mediating oxidative stress induced by high glucose[31]. In T2DM, the expression of p66Shc in peripheral blood monocytes was also found to be significantly increased[32], and the transcription of p66Shc mRNA was enhanced in human umbilical vein endothelial cells cultured with high glucose *in vitro*[33]. Activated p66Shc can translocate from the cytoplasm to mitochondria, oxidize cytochrome c, induce ROS production, and ultimately cause oxidative damage and cell apoptosis. It further promotes the phosphorylation of insulin receptor inhibitor-1, activates mammalian target of rapamycin receptor ribosome S6 protein kinase, leads to IR and exacerbates DM progression[34]. Thus, p66Shc plays an important role in the pathogenesis of DM.

However, the role of p66Shc in GDM remains unclear. In this study, we found that the expression of p66Shc was increased in the peripheral blood and placentas of patients with GDM and was significantly higher in JEG3 cells treated with high glucose than in those treated with low glucose *in vitro*. Compared with healthy pregnant women, our data showed that the expression of Drp1 and the level of ROS in GDM patients increased when the expression of p66Shc was increased, but decreased when p66Shc was decreased. Therefore, the activity of Drp1 and ROS may be regulated by p66Shc in the placentas of patients with GDM. This reveals that the increased expression of p66Shc was involved in high-glucose induced oxidative stress in human trophoblast cells and placentas. In addition, the expression of p66Shc and Drp1 in JEG3 cells under high glucose conditions was time dependent, showing that mitochondrial damage increased over time. Therefore, mitochondrial damage and ROS in patients with poor glycemic control may be increased during the development of GDM, and the possibility of pregnancy complications will also increase.

To date, the relationship among p66Shc, Drp1 and ROS in GDM is still unclear. Some studies have reported that p66Shc inhibited mitochondrial division in several types of cells, such as neuronal cells and fat-derived stem cells, thereby promoting cell damage and apoptosis[35,36]. In addition, the ability of p66Shc to alter the mitochondrial crista morphology of immune T cells has also been reported[37]. Drp1, a gene that promotes mitochondrial division, is a dynein necessary for mitochondrial division. The concept that mitochondrial fragments are prerequisites for the production of ROS has recently been proposed[38]. In DM, mitochondrial debris and ROS interact, creating a vicious cycle. Based on previous research, we found that GDM involved mitochondrial oxidant stress generation, perturbation of mitochondrial dynamics and mitochondrial fragmentation. In this study, we observed a significant increase in the expression of Drp1 and the levels of ROS in JEG3 cells overexpressing activated p66Shc. In contrast, p66Shc knockdown reduced the expression of Drp1 and the level of ROS. Therefore, several lines of evidence have demonstrated that p66Shc is a crucial mediator of mitochondrial dysfunction. This result shows that high glucose promoted mitochondrial fragmentation following p66Shc activation and excessive ROS, which may further cause mitochondrial damage and cell apoptosis, contributing to the occurrence and development of GDM. Our study investigated the potential mechanism of p66Shc in GDM *in vitro*, finding that p66Shc may be an important molecule in the development of GDM, which needs to be confirmed in large clinical studies. This study may provide a new molecular mechanism and experimental basis for the role of mitochondrial damage in the pathogenesis of gestational diabetes mellitus and provide a new approach for the treatment of GDM and its complications.

CONCLUSION

The expression of p66shc in peripheral blood and the levels of p66shc and Drp1 in placental tissues were significantly increased in patients with GDM. The increased expression of p66Shc induced by high glucose-activated Drp1 and promoted ROS overproduction, which may be the primary cause of cell damage and apoptosis during the occurrence and development of GDM. This study preliminarily explored the relationships of p66Shc, mitochondria, and oxidative stress in GDM, providing new ideas and evidence regarding the etiology and treatment of GDM.

ARTICLE HIGHLIGHTS

Research background

Oxidative stress and mitochondrial dysfunction in the placenta are closely related to the onset of gestational diabetes mellitus (GDM). p66Shc plays a role in regulating mitochondrial oxidative stress, and dynamin-related protein 1 (Drp1) is a necessary dynamic protein for mitosis of primarily localized mitochondria.

Research motivation

The motivation was to add to what is known of the role of placental mitochondria in the etiology of GDM.

Research objectives

The study aimed to investigate the potential mechanism of p66Shc in GDM.

Research methods

We detected the expression of Drp1 and p66Shc in patients with GDM and investigated the possible pathogenesis of GDM through *in vitro* culture of the JEG3 human trophoblast line.

Research results

P66Shc, Drp1, and reactive oxygen species (ROS) were highly expressed in the placentas and peripheral blood during GDM and in JEG3 cells under high glucose conditions. A significant increase in the expression of Drp1 and the level of ROS was detected in JEG3 cells overexpressing activated p66Shc. In contrast, p66Shc knockdown reduced the expression of Drp1 and the level of ROS.

Research conclusions

The increased expression of p66Shc induced by high glucose-activated Drp1 and promoted ROS overproduction, which may contribute to the occurrence and development of GDM.

Research perspectives

This study may provide a new understanding of molecular mechanism and experimental basis for the role of mitochondrial damage in the pathogenesis of gestational diabetes mellitus and provide a new approach for the treatment of the condition and its complications.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Factors influencing the effectiveness of using flash glucose monitoring on glycemic control for type 1 diabetes in Saudi Arabia

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Abstract

BACKGROUND

In 2017, 35000 Saudi children and adolescents were living with a type 1 diabetes (T1D) diagnosis. Diabetic complications are minimized upon strengthened glycemic regulation. The annual cost of treating diabetic patients with complications was four-fold higher than for patients without complications. The use of flash glucose monitoring (FGM) enables better diabetes treatment and thereby improves glycemic control. Understanding the factors that affect effectiveness of FGM will help enhance the device's use and management of hospital resources, resulting in improved outcomes.

AIM

To investigate factors that affect effectiveness of the FGM system for glycated hemoglobin (HbA1c) levels/glycemic control among T1D patients.

METHODS

A retrospective empirical analysis of T1D patient records from King Abdul-Aziz University Hospital and Prince Sultan Military Medical City was performed. T1D patients who began FGM between 2017 and 2019 were included.

RESULTS

The data included 195 T1D patients (70 males and 125 females) with a mean age of 23.6 ± 8.1 years. Among them, 152 patients used multiple daily injection and 43 used an insulin pump. The difference in HbA1c level from baseline and after using FGM was -0.60 ± 2.10, with a maximum of 4.70 and a minimum of -6.30. There was a statistically significant negative correlation between the independent variables (age, duration of diabetes, level of engagement) and HbA1c. The group with the highest HbA1c mean (9.85) was 18-years-old, while the group with the lowest HbA1c mean (7.87) was 45-years-old. Patients with a low level of

engagement (less than six scans per day) had the highest HbA1c mean (9.84), whereas those with a high level of engagement (more than eight scans per day) had the lowest HbA1c mean (8.33).

CONCLUSION
With proper education, FGM can help people with uncontrolled T1D over the age of 18 years to control their glucose level.

Key Words: Type 1 diabetes; Flash glucose monitoring; Continuous glucose monitoring; Hypoglycemia; Glycemic control

Core Tip: The factors influencing success of flash glucose monitoring are poorly understood in people with type 1 diabetes (T1D). Therefore, we retrospectively investigated the main predictor factors and their relationship with glycemic control/glycated hemoglobin (HbA1c) levels in 195 patients with T1D. Flash glucose monitoring resulted in a clinically significant reduction in HbA1c, and the uncontrolled group (baseline HbA1c > 9) had the highest reduction in HbA1c. Age and level of engagement were negatively associated with HbA1c. Patients in the age group (18-45 years) with a high level of engagement were more likely to experience a higher-reduction in HbA1c.

INTRODUCTION

In 2017 alone, 35000 Saudi children and adolescents (aged 20 years) were living with a type 1 diabetes (T1D) diagnosis, according to a study from the International Diabetes Federation, with 3900 new cases yearly[1]. Uncontrolled diabetes has an effect on nearly every organ in the body, and good glycemic control lowers the risk of diabetic complications. While the cost of treating diabetic patients with complications was US$ 11706.90 per year, this was reduced to US$ 2746.70 per year for diabetic patients without complications[2]. According to the American Diabetes Association, diabetic patients on multiple daily injections (MDIs) or insulin pumps can monitor blood glucose levels before meals, postprandially, at bedtime, before exercise, when they suspect hypoglycemia, after treating hypoglycemia, and before performing vital activities such as driving[3]. Increasing the number of times one monitors their blood glucose level is linked to lower glycated hemoglobin (HbA1c) levels and fewer complications[4].

The new continuous glucose monitoring devices and flash glucose monitoring (FGM) provide reliable glucose readings for up to 14 d after a 1 h warm-up cycle. This consists of two main components: a portable reader and a disposable, factory-calibrated sensor worn on the back of the upper arm by the patient. The reader is used by the patient to wirelessly scan the sensor and obtain glucose readings. Every minute a sensor measures the glucose concentration in the interstitial fluid. It also automatically records the glucose concentration every 15 min and saves the information in an 8-h log. The use of this technology has a beneficial influence on patient adherence to blood glucose monitoring and glycemic control[2] because it accurately measures interstitial fluid glucose within a reasonable range of error as capillary blood glucose [5-9]. FGM is more costly than standard treatment, and there are no set guidelines for which patients should use it and when they should start using it.

Understanding the factors that affect the effectiveness of FGM will help enhance the device's use and management of hospital resources, resulting in improved outcomes. Research has shown that using continuous glucose monitoring improves glycemic

control by lowering HbA1c levels, reducing the number of hypoglycemic events, increasing time in the target range and reducing the number of hospital visits due to hypoglycemia or ketoacidosis[4,10-12].

MATERIALS AND METHODS

The formula of Hulley *et al*[13] was used to determine the sample size. Accordingly, a sample size of 195 patients was used in the current study. This study was a retrospective study involving 234 patients who had undergone FGM during the study period from the involved research centers (60 patients from King Abdulaziz University Hospital and 174 from Prince Sultan Military Medical City). Patients who were 15 years or older with T1D, used FGM for at least 1 mo, and were capable of checking and controlling their glucose levels themselves based on the data generated by the sensor were included in the study. However, 39 people requiring tighter glycemic control were excluded from the study (13 had type 2 diabetes, 6 had chronic kidney disease, 6 were under the age of 15, and 10 were new to FGM). In the present study, the dependent variable was HbA1c, while the independent variables were age, body mass index (BMI), diabetes length, duration of using FGM, degree of involvement, and type of insulin treatment. Demographic information as well as lab results were extracted from each hospital's information system. Because each patient's sensor data was stored in their register, the artificial intelligence was able to derive the degree of commitment, which was determined by the average number of scans per day. Ethical approval was obtained from the King Saud University ethics committee (Ref No: 19/0812/IRB) as well as access letters from both hospitals. Data collection lasted for about 4 wk.

Statistical analysis

Statistical analysis was performed using SPSS software (Version 23; IBM Corp., Armonk, NY, United States). The dependent variable (HbA1c) was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests across the independent variables. Differences between groups for unevenly distributed data were analyzed using non-parametric tests (Mann-Whitney test, Kruskal-Wallis test). For the association, Spearman's correlation coefficient was used. Independent sample *t*-tests were used for data with two groups (duration of using FGM and type of insulin treatment), and one-way analysis of variance was used for data with three or more groups. Pearson's correlation coefficient was used for the relationship of data with more than two groups (duration of diabetes and level of engagement). The relationship between the type of insulin treatment (nominal data) and the dependent variable HbA1c was analyzed using cross tabulation.

RESULTS

The demographic characteristics of the included patients are represented in Table 1. The result shows that the average age of the patients was 23.6 years, with almost half (49.7%) of them being between the ages of 18 and 30. The majority of the participants were women (64.1%). In terms of engagement, the majority of patients (48.7%) had a low level of engagement, scanning fewer than six times per day. MDI was chosen as the type of insulin treatment by 77.9% of the participants.

Using the Wilcoxon signed rank test, FGM resulted in a statistically significant reduction in HbA1c (z = -4.535, P = 0.000) with a broad effect size (r = 0.119). From pre-FGM (median = 9.7) to post-FGM (median = 9.0), the median HbA1c score decreased. In 62% of the patients, HbA1c was reduced after FGM, and the majority of them (76.0%) had an HbA1c of more than 9. On the other hand, HbA1c levels increased in 32% of patients, and 39% of them had an HbA1c of less than 8. In 11 patients, there was no difference in HbA1c. The highest rise in HbA1c was 4.7%, while the maximum decrease was 6.3%.

Relationship between age and HbA1c

The relationship between age and HbA1c was moderately negative and statistically significant (r_s = -0.373, P = 0.000). Kruskal-Wallis test showed a statistically significant difference in mean HbA1c between the age groups (P = 0.001, χ^2 = 17.79). The age group under 18 years had the highest HbA1c mean level (9.8 ± 1.5), while the age

Table 1 Descriptive statistics of patient characteristics, *n* = 195

Parameter		Count[1]	%[1]
Age in yr, mean ± SD: 23.6 ± 8.1	< 18	60	30.8
	18-30	97	49.7
	31-45	35	17.9
	> 45	3	1.5
Sex	Male	70	35.9
	Female	125	64.1
Level of engagement	< 6 times/d	95	48.7
	6-8 times/d	34	17.4
	> 8 times/d	66	33.8
Type of insulin treatment	MDI	152	77.9
	Insulin pump	43	22.1

[1]Descriptive analysis (frequencies and percentages).
MDI: Multiple daily injection.

group over 45 years had the lowest level (7.8 ± 0.8) (Figure 1A).

Relationship between BMI and HbA1c
Although BMI and HbA1c had a weak negative correlation (r_s = -0.129, P = 0.068), the correlation was not statistically significant. There was no statistically significant difference in HbA1c between the different BMI categories according to the Kruskal-Wallis test [P = 0.141, χ^2 (2) = 5.461] (Figure 1B).

Relationship between duration of diabetes and HbA1c
There was a weak negative relationship between diabetes duration and HbA1c, which was statistically significant (r_s = -0.162, P = 0.024). A one-way analysis of variance test revealed no statistically significant difference in HbA1c levels between groups of diabetes duration [P = 0.231, F (4,190) = 4.168] (Figure 1C).

Relationship between level of engagement and HbA1c
The relationship between degree of involvement and HbA1c was moderately negative and statistically significant (r = -0.394, P = 0.000). One-way analysis of variance test showed a statistically significant difference in mean HbA1c between the various levels of engagement (P = 0.000, F = 17.733). The HbA1c level after FGM was significantly lower in those who scanned six to eight times per day (8.9 ± 1.5, P = 0.018) and those who scanned more than eight times per day (8.3 ± 1.3, P = 0.000) compared to those who scanned less than six times per day (9.8 ± 1.7) (Figure 1D).

Relationship between type of insulin treatment and HbA1c
The form of insulin therapy had a mild relationship with HbA1c, and the correlation was statistically significant (η^2 = 0.094, P = 0.000). The MDI group and the insulin pump group had a statistically significant difference in mean HbA1c according to an independent-sample *t*-test. The MDI group had a higher mean HbA1c (9.5 ± 1.7) than the insulin pump group (8.2 ± 1.2) (P = 0.000, t = 4.49) (Figure 1E).

Relationship between duration of using FGM and HbA1c
The length of FGM use and HbA1c had a weak negative relationship, but the correlation was not statistically important (r = -0.116, P = 0.107). Using an independent-sample *t*-test, no statistically significant difference in mean HbA1c was found between the two classes of FGM duration [t (195) = 1.57, P = 0.765]. Patients who used FGM for less than 3 mo had an HbA1c of 9.4 ± 1.7 compared to those who used it for more than 3 mo, who had an HbA1c of 9.0 ± 1.8 (Figure 1F).
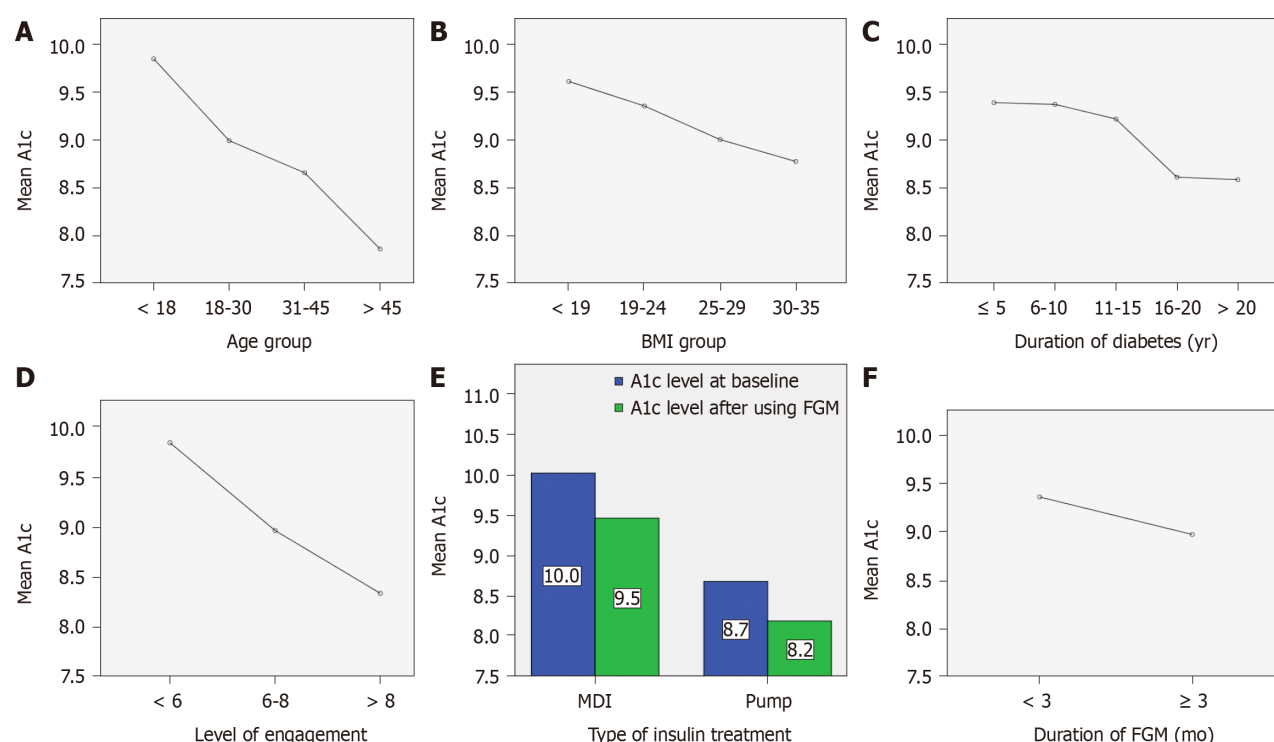
Figure 1 Relationship among various factors. A: Relationship between age and mean glycated hemoglobin levels; B: Relationship between body mass index and glycated hemoglobin levels; C: Relationship between duration of diabetes and glycated hemoglobin levels; D: Relationship between level of engagement and glycated hemoglobin levels; E: Relationship between type of insulin treatment and glycated hemoglobin levels; F: Relationship between duration of using flash glucose monitoring and glycated hemoglobin levels. A1c: Glycated hemoglobin; BMI: Body mass index; FGM: Flash glucose monitoring; MDI: Multiple daily injection.

DISCUSSION

Several medical associations are now taking essential steps to help patients with T1D and type 2 diabetes regulate their glycemic index. Monitoring the amount of glucose in a diabetic patient's body is an important part of diabetes management and the treatment process. FGM is an excellent method for assessing the level of "glucose metabolic disturbance" and directing the treatment process. Diabetes affects a large number of children in Saudi Arabia. As a result, FGM instruments are currently used to calculate the amount of glucose in the blood. This is achieved by implanting a tracker under the skin for 14 d to monitor blood glucose, and the patient must check the sensor's reader and read the blood glucose level over the previous 8 h.

FGM is expensive to use because one sensor costs $89 and test strips cost $0.75 each. The National Health Service has developed some guidelines for those who are eligible to receive funding for FGM. These individuals must be on "intensive insulin therapy" and plan to attend an education session on the topic of FGM, be able to scan their glucose levels at least six times a day, report their findings with the National Health Service clinic, and participate in a "diabetes self-management" education program [14]. Determining the effectiveness of FGM will help to justify the cost in patients who will receive the greatest benefit.

In the present study, FGM resulted in a substantial and clinically significant reduction in HbA1c. This result confirms findings from previous studies that looked at the efficacy of FGM in diabetic patients[15-17]. In the current study, HbA1c was decreased by at least 0.5 in 104 patients, with 84 of them having a baseline HbA1c of more than 9. After using FGM, the uncontrolled group had the greatest reduction in HbA1c levels at baseline, while the monitored group had no reduction or an increase in HbA1c. This is in line with the findings of Tyndall *et al*[14] who used FGM on 900 T1D patients and followed them for 245 d. According to the findings of their report, some patients' HbA1c levels changed. Individuals who did not use FGM had no improvement in their HbA1c during the same time span. The change in A1c was the study's primary outcome.

Patients under the age of 18 years had the smallest change in HbA1c levels after using FGM, out of all age groups. Our results in the present study confirm those from a previous study that showed that HbA1c levels in patients under the age of 18 worsen over time[18]. Patients in the 18-45 years age group showed the greatest decrease in

HbA1c levels relative to other age groups. This was consistent with the findings from the Paris *et al*[19] study, which was conducted to determine the effectiveness of FGM on HbA1c in 120 T1D patients between the ages of 18-76 years. The retrospective study reported positive improvements in HbA1c, with FGM being especially helpful for patients with a baseline HbA1c of 7.5%.

According to the study by Campbell *et al*[20] of 76 participants, including children and adolescents, FGM supported children with T1D with an average HbA1c reduction of 0.4 (from 7.9 to 7.5), indicating that patients with T1D who used FGM had better glycemic control. Just 13 patients (6.1%) in the current study began FGM within the first year of diagnosis, which was used to compare early and late initiation of FGM after diagnosis. A significant but weak correlation between late initiation and lower HbA1c was observed, which contradicts the findings from a previous study that looked at the usability and efficacy of starting FGM within the first year of T1D diagnosis. That study observed that patients who began FGM earlier had greater glycemic regulation than those who started FGM later[11]. Anderson and his colleagues[21] contrasted the HbA1c levels between long-term and short-term usage of continuous glucose monitoring in 10 outpatient clinics over a 1.1 year period in a retrospective sample and observed that long-term users had lower HbA1c levels than short-term users, which was statistically significant. In regard to patients who were on insulin pump treatment, 41.8% had baseline HbA1c < 8 compared to only 19.7% of patients in the MDI group. This may have influenced the observed association between HbA1c levels after FGM and the insulin pump group. Over a 2.5 year duration, Mulinacci *et al*[11] observed in 396 new T1D patients that MDI patients with FGM had a 1.5% lower HbA1c compared to MDI patients without FGM. Patients who were treated with an insulin pump and started on FGM had a 0.7% lower HbA1c than those who were on an insulin pump but not on FGM. The study concluded that, regardless of insulin treatment type, early use of FGM was helpful in lowering blood glucose levels in T1D patients.

Because the sensor measures interstitial fluid glucose, the accuracy of FGM is inversely related to BMI, which can ultimately affect the HbA1c level[22]. Using 58 T1D patients aged 18-years-old to 64-years-old, the accuracy of interstitial glucose was compared to FGM. Two sensors were implanted in each participant, and a record was taken at 10 h, 12 h, 24 h and 72 h after the insertion. The results showed the median and mean absolute relative difference values were 9.3% and 12.8%, respectively. The study observed that FGM sensor measurements were as reliable as the venous measurements, but that BMI had a minor impact on accuracy[23]. The accuracy of the FGM sensor was not affected by BMI in the Bailey *et al*[24] report, which is consistent with what was observed in the current study. We did not identify any correlation between BMI and HbA1c.

High levels of interaction resulted in lower HbA1c levels, which was consistent with previous studies[19,25,26]. The 12 mo observational study involving 120 people conducted by Paris *et al*[19] to determine FGM use in T1D patients with frequent hypoglycemia showed that HbA1c levels improved after 3 mo of FGM use in certain patients. The study also found a clear association between HbA1c and scanning frequency, which matches the results of the current study. As a result, patient education is crucial in motivating FGM users to scan at least six times per day to collect 100% of the data (before and after meals, before and after exercise, and before sleep).

This study had some limitations. While retrospective analysis helps us to collect data over a longer period of time, it is impossible to monitor the confounding factors that can influence HbA1c levels with this type of study design. Another significant drawback of this study was the lack of FGM sensor availability during the research period. Due to registration issues with a few patients, it was not possible to obtain the most recent HbA1c readings. The number of people involved in the sample was not equal because each hospital had different requirements for starting a diabetic patient on FGM.

CONCLUSION

FGM is an acceptable technology for T1D patients over the age of 18 years who are committed to monitoring their glucose levels at least six times a day because it offers real-time information. As a result, it can assist patients in maintaining glucose regulation by making the right decisions. FGM is a secure procedure with a high level of consumer acceptance in real-life situations.

ARTICLE HIGHLIGHTS

Research background

Type 1 diabetes (T1D) affects a large number of children and adolescents in Saudi Arabia, and as a result flash glucose monitoring (FGM) devices are widely used. The factors influencing the effectiveness of FGM are poorly understood in people with T1D.

Research motivation

FGM is more expensive than standard treatment, and there is no guideline for which patients should receive FGM or when they should start FGM. Each hospital in Saudi Arabia has different requirements for starting a diabetic patient on FGM. The effectiveness of FGM can be influenced by many factors, including age, body mass index, type of insulin treatment, duration of diabetes, duration of using FGM, and level of patient engagement.

Research objectives

We investigated the association between glycated hemoglobin (HbA1c) levels after using FGM and potential predictor factors in a population with T1D. The ultimate goal was to help develop national guidelines for those who are eligible to receive funding for FGM, which in turn will enhance the utilization of the device and manage hospital resources, resulting in improved outcomes.

Research methods

In this retrospective cohort study of 195 T1D patients aged 15 years and above who had used FGM for at least 1 mo, demographic and clinical parameters and related data were extracted from patient records at two hospitals.

Research results

FGM in this study resulted in a clinically significant reduction in HbA1c (-0.6 ± 2.1). The uncontrolled group (baseline HbA1c > 9) had the largest reduction in HbA1c levels. There was a statistically significant moderate and negative association between age and level of engagement and HbA1c levels. Patients in the age group of 18-years-old to 45-years-old with a high level of engagement were more likely to demonstrate a large reduction in HbA1c levels. The relationships between HbA1c and other factors varied between no association to weak association.

Research conclusions

FGM is a more effective technology for T1D patients over the age of 18 years who are committed to checking their glucose level at least six times a day.

Research perspectives

To identify the relationships between HbA1c levels and predictor factors on the long-term use of FGM, a multicenter, prospective, large-scale study on patients with T1D should be conducted in the future.

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ORIGINAL ARTICLE

Observational Study

Association between admission hemoglobin level and prognosis in patients with type 2 diabetes mellitus

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Abstract

BACKGROUND
Anaemia is common in patients with chronic kidney disease (CKD) and is a major risk factor that contributes to mortality in such patients. Type 2 diabetes mellitus (T2DM) is one of the leading causes of CKD. The association between admission hemoglobin levels and renal damage in patients with T2DM remains unclear.

AIM
To evaluate the relationship between admission hemoglobin levels and prognosis in patients with T2DM.

METHODS
We performed a retrospective analysis of 265 consecutive patients presenting with T2DM between 2011 and 2015. The composite endpoint was end-stage renal disease or a 50% reduction in the estimated glomerular filtration rate.

RESULTS
In multivariable-adjusted Cox proportional hazards models (adjusting for demographic factors, traditional risk factors, lipids), the adjusted hazard ratios (HRs) for the highest and middle tertiles compared to the lowest tertile of hemoglobin were 0.82 (95%CI: 0.11-6.26, $P = 0.8457$) and 0.28 (95%CI: 0.09-0.85, $P = 0.0246$), respectively. However, after further adjustment for glycaemia control, hemoglobin was positively related to the risk of the composite endpoint (HR: 1.05, 95%CI: 0.14-8.09, $P = 0.9602$) when the highest tertile was compared to the lowest tertile of hemoglobin. We found a U-shaped relationship between hemoglobin levels and the composite endpoint. The curve tended to reach the lowest level at

an optimal hemoglobin level.

CONCLUSION

Among patients with T2DM, a U-shaped relationship was observed between hemoglobin levels and renal damage. A lower admission hemoglobin level (hemoglobin < 13.3 g/dL) is an independent predictor of renal damage.

Key Words: Type 2 diabetes mellitus; Hemoglobin; Renal damage; Prognosis

Core Tip: A U-shaped exposure-response relationship exists between admission hemoglobin levels and the composite endpoint among patients with type 2 diabetes mellitus. A lower admission hemoglobin level (hemoglobin < 13.3 g/dL) is an independent predictor of renal damage. Hemoglobin is a convenient and feasible way to identify those patients who are at high risk of having a poor prognosis.

INTRODUCTION

Anaemia is a common complication of chronic kidney disease (CKD). Low hemoglobin level is one of the major risk factors for cardiovascular disease (CVD) and mortality and poor prognosis in CKD patients[1-4]. However, some studies have shown that higher hemoglobin levels slightly increase the risk of death[5], and elevations in hemoglobin levels have been implicated in a higher risk of mortality and cardiovascular events[6,7]. Information on the association of hemoglobin and renal damage is uncertain, and the optimal hemoglobin target in CKD patients remains controversial. Type 2 diabetes mellitus (T2DM) is one of the leading causes of CKD. The admission hemoglobin levels would influence the prognosis in patients with T2DM. In the present study, we aimed to evaluate the relationship between admission hemoglobin levels and the prevalence of renal damage and the decline in the estimated glomerular filtration rate (eGFR) in patients with T2DM. Our hypothesis was that there may be an optimal hemoglobin level about the relationship between hemoglobin and renal progression in patients with T2DM. This study may help us to find the optimal hemoglobin level. And the optimal hemoglobin level may be used as an intervention target for patients with T2DM.

MATERIALS AND METHODS

This was a retrospective cohort study. The details of this study were described previously[8]. We used the database from the Shenzhen Second People's Hospital and reviewed the records. A total of 265 patients diagnosed with T2DM were enrolled between January 2011 and December 2015 and followed until June 2016 at the Department of Nephrology and Endocrinology of Shenzhen Second People's Hospital. The patients were followed every 3 mo for at least 3 mo until study endpoint or deadline. The deadline for the study was June 30, 2016. Patients with moderate to severe valvular disease, atrial fibrillation, other severe arrhythmias, congenital heart disease, or primary myocardial disease were excluded. The patients who had missing data for the admission hemoglobin levels and the composite endpoint were also excluded.

By using the database from the Shenzhen Second People's Hospital, general clinical data, including age, gender, duration of T2DM, history of hypertension, use of angiotensin-converting enzyme inhibitors (ACEI) and/or angiotensin receptor

blockers (ARB), body mass index (BMI; kg/m²), systolic blood pressure (SBP), and diastolic blood pressure (DBP), were recorded. Laboratory evaluations included fasting glycaemia, glycosylated hemoglobin (HbA1c), serum creatinine (Scr), 24-h urinary protein, hemoglobin, serum albumin (ALB), serum uric acid, blood urea nitrogen, total cholesterol (TC), and triglycerides (TG). All these laboratory tests were performed and checked at the Central Laboratory of Shenzhen Second People's Hospital. The blood and urine samples were collected when patients were on admission. And the blood samples were fasting venous blood. Urine samples were collected in one or more containers over a period of 24 h. Reagent based method and automated analyzer were used to measure these laboratory variables. The eGFR was calculated using the CKD epidemiology collaboration equation[9]. The stages of CKD were classified as follows[10]: Stage 1, kidney damage with normal or increased eGFR (> 90 mL/min/1.73 m²); stage 2, mild reduction in eGFR (60-89 mL/min/1.73 m²); stage 3, moderate reduction in eGFR (30-59 mL/min/1.73 m²); stage 4, severe reduction in eGFR (15-29 mL/min/1.73 m²); stage 5, kidney failure (eGFR < 15 mL/min/1.73 m² or dialysis). Our focus was to understand the effect of hemoglobin on the composite endpoint.

Follow-up and endpoint

The patients were followed every 3 mo for at least 3 mo until June 2016. The composite endpoint was end-stage renal disease or a 50% reduction in the eGFR.

Statistical analysis

The Shapiro–Wilk test was used to test the normality of the data. Continuous variables are expressed as the mean ± SD (normal distribution) or median (quartiles) (skewed distribution), and categorical variables are expressed as frequencies or percentages. One-way ANOVA (normal distribution), Kruskal-Wallis H (skewed distribution) test, and chi-square test (categorical variables) were used to determine any significant differences between the means and proportions of the groups. The effects of hemoglobin levels on the composite endpoint were evaluated using Cox proportional hazards regression without adjustment and with adjustment for confounding variables. Hazard ratios (HRs) were reported per g/dL increment of hemoglobin levels (g/dL). Incremental models were fitted adjusting for (model 1) demographic factors (age, gender, and BMI); (model 2) demographic and traditional renal function risk factors (baseline eGFR, history of hypertension, SBP, DBP, 24-h urinary protein, ACEI and/or ARB use, and ALB); (model 3) demographic factors, traditional risk factors, and lipids (TC and TG); and (model 4) demographic factors, traditional risk factors, lipids, and glycaemia control (duration of diabetes, fasting glycaemia, and HbA1c). Kaplan–Meier plots were generated using tertiles of hemoglobin data to illustrate findings. A smoothing spline curve was used to describe the adjusted relationship between hemoglobin levels and the composite endpoint. Two-tailed probability values of < 0.05 were considered to indicate statistical significance. All statistical analyses were performed using Empowerstats (www.empowerstats.com) and R software (http://www.R-project.org).

Declarations

The proposal was approved by the Clinical Research Ethical Committee of the Shenzhen Second People's Hospital, and all subjects provided informed consent before enrollment. We adhered to the principles of the Declaration of Helsinki. The procedures followed were in accordance with institutional guidelines.

RESULTS

Association of hemoglobin levels with demographic and clinical factors

The average hemoglobin for the whole sample was 12.77 ± 2.42 g/dL. The distribution of hemoglobin levels in the full cohort is shown in Figure 1. We categorized the admission hemoglobin levels into tertiles to analyze. The cut-points for the tertile 1 (T1), tertile 2 (T2), and tertile 3 (T3) were < 11.97, 11.97-< 13.90, and 13.90-< 19.40, respectively. The baseline characteristics of study participants by hemoglobin tertiles are presented in Table 1. There were significant associations of higher hemoglobin with decreasing age, duration of diabetes, SBP, BUN, potassium, phosphate, HbA1c, 24-h urinary protein, and less history of hypertension. Male sex, ACEI/ARB use, and higher BMI, eGFR, DBP, ALB, and fasting blood glucose were also associated with

Table 1 Baseline characteristics of study participants according to hemoglobin levels

Variable	Hemoglobin tertile-Total	Hemoglobin tertile-Tertile 1 (< 11.97)	Hemoglobin tertile-Tertile 2 (11.97-< 13.90)	Hemoglobin tertile-Tertile 3 (13.90-< 19.40)	*P* value
Male, *n* (%)	166 (62.64)	34 (38.64)	49 (59.04)	83 (88.30)	< 0.001
Female, *n* (%)	99 (37.36)	54 (61.36)	34 (40.96)	11(11.70)	< 0.001
Age, years	58.08 ± 12.37	61.67 ± 11.89	59.23 ± 10.35	53.69 ± 13.22	< 0.001
BMI, kg/m^2	25.18 ± 3.22	24.55 ± 3.40	24.94 ± 2.97	25.98 ± 3.10	0.010
Duration of diabetes, months	33.54 ± 63.81	47.14 ± 79.51	37.60 ± 66.98	17.22 ± 35.07	0.005
eGFR, mL/min *per* 1.73 m^2	90.88 ± 46.71	60.63 ± 47.85	95.80 ± 41.82	114.85 ± 32.26	< 0.001
History of hypertension	171 (64.53%)	64 (72.73%)	57 (68.67%)	50 (53.19%)	0.014
SBP, mmHg	141.78 ± 23.10	148.42 ± 26.85	141.14 ± 22.07	136.12 ± 18.33	0.001
DBP, mmHg	79.11 ± 12.51	75.66 ± 13.83	79.69 ± 10.50	81.84 ± 12.21	0.003
ALB, g/L	39.54 ± 5.59	35.98 ± 5.61	40.03 ± 5.20	42.46 ± 3.80	< 0.001
BUN, mmol/L	7.47 ± 6.24	9.79 ± 5.99	7.61 ± 8.49	5.15 ± 1.73	< 0.001
SUA, µmol/L	401.18 ± 250.95	449.22 ± 403.60	384.23 ± 106.65	370.82 ± 109.02	0.085
Potassium, mmol/L	4.30 ± 1.00	4.55 ± 1.26	4.31 ± 1.04	4.05 ± 0.50	0.003
Total calcium, mmol/L	2.31 ± 0.47	2.28 ± 0.56	2.35 ± 0.49	2.30 ± 0.34	0.619
Phosphate, mmol/L	1.25 ± 0.31	1.32 ± 0.34	1.28 ± 0.32	1.16 ± 0.24	0.002
Fasting glycaemia, mmol/L	7.95 ± 3.26	7.30 ± 3.09	7.65 ± 3.30	8.83 ± 3.22	0.004
HbA1c, %	11.40 ± 28.88	15.10 ± 49.59	10.22 ± 12.89	9.21 ± 2.13	0.015
TG, mmol/L	2.00 ± 1.71	1.83 ± 1.50	1.69 ± 1.24	2.41 ± 2.12	0.014
TC, mmol/L	4.52 ± 2.28	4.45 ± 1.53	4.70 ± 3.64	4.42 ± 0.96	0.703
24-h urinary protein, mg/d	1179.17 ± 2346.72	2345.71 ± 3176.33	966.77 ± 2086.06	274.63 ± 450.60	< 0.001
ACEI/ARB use, *n* (%)	228 (86.04)	58 (65.91)	77 (92.77)	93 (98.94)	< 0.001
CKD stage, *n* (%)					< 0.001
1	148 (55.85)	23 (26.14)	51 (61.45)	74 (78.72)	
2	41 (15.47)	10 (11.36)	14 (16.87)	17 (18.09)	
3	38 (14.34)	25 (28.41)	12 (14.46)	1 (1.06)	
4	19 (7.17)	13 (14.77)	4 (4.82)	2 (2.13)	
5	19 (7.17)	17 (19.32)	2 (2.41)	0 (0.00)	

Continuous variables are presented as the mean (SD) or median (IQR), and categorical variables are presented as the number (percentage). BMI: Body mass index; eGFR: Estimated glomerular filtration rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; ALB: Serum albumin; BUN: Blood urea nitrogen; SUA: Serum uric acid; TC: Total cholesterol; TG: Triglycerides; ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blockers; CKD: Chronic kidney disease.

higher hemoglobin.

Association between hemoglobin levels and the composite endpoint

During the follow-up period of 15.19 ± 9.33 mo, a total of 52 participants experienced the composite endpoint. Study entry hemoglobin levels independently and inversely correlated with the risk of the composite endpoint (HR: 0.74, *P* = 0.0012) in the model adjusted for demographic factors and known risk factors. The composite endpoint remained significant (HR: 0.65, *P* = 0.0055) after further adjustment for lipids and glycaemia control (Figure 2).
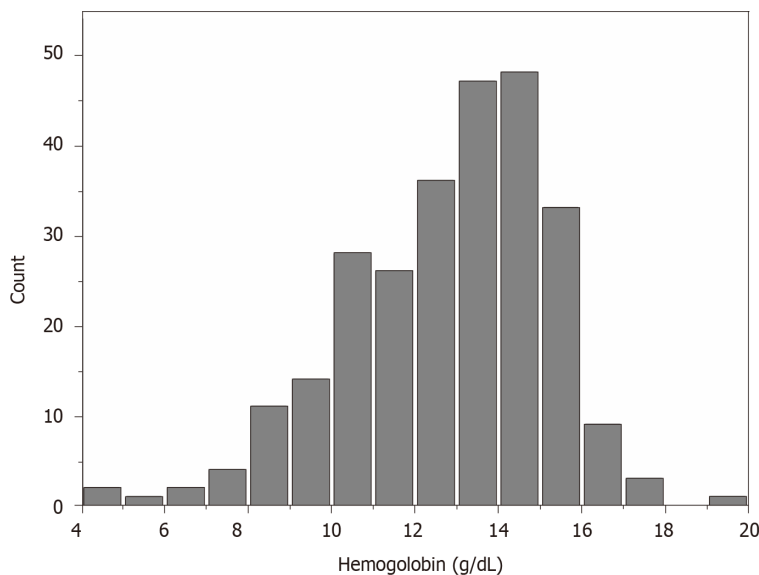
Figure 1 Distribution of hemoglobin levels in the study sample.
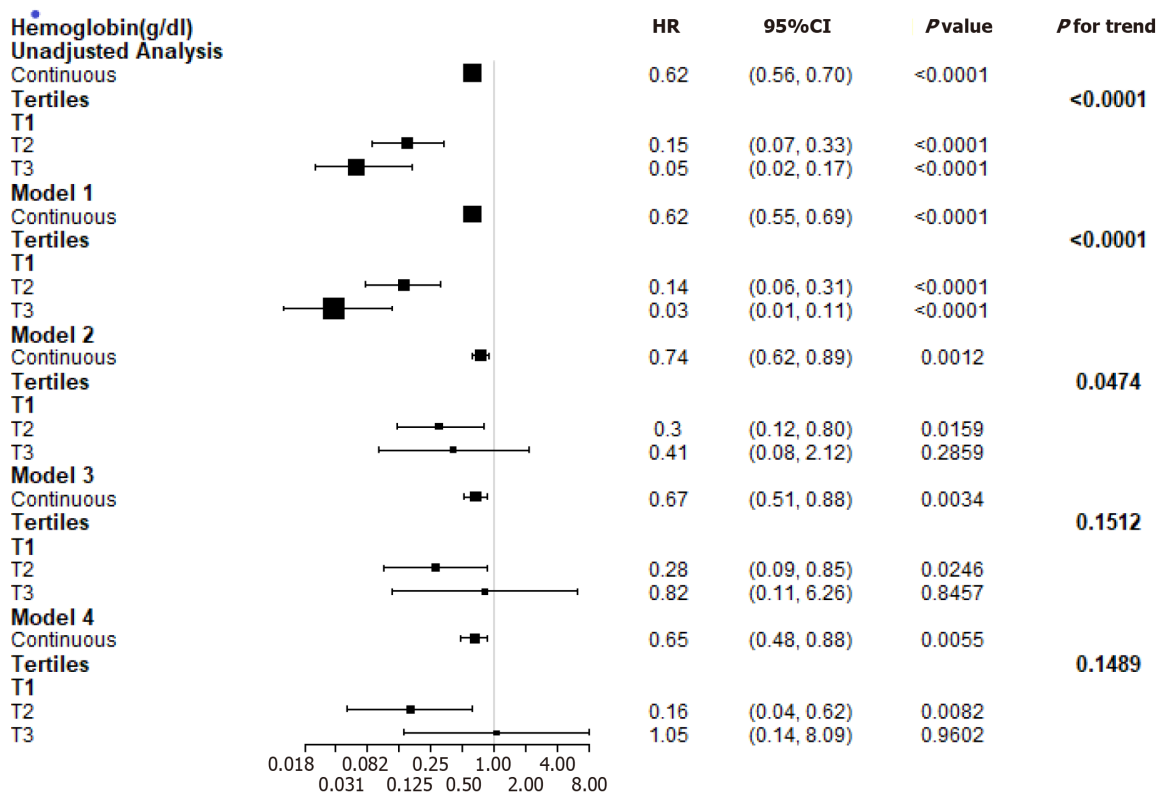
Figure 2 Estimated hazard ratio for the composite endpoint associated with hemoglobin in different models. Model 1: Demographic factors (age, gender, and body mass index); model 2: Demographic factors and traditional renal function risk factors (baseline estimated glomerular filtration rate, history of hypertension, systolic blood pressure, diastolic blood pressure, 24-h urinary protein, angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers use, and albumin); model 3: Demographic factors, traditional risk factors, and lipids (total cholesterol and triglycerides); model 4: Demographic factors, traditional risk factors, lipids, and glycaemia control (duration of diabetes, fasting glycaemia, and glycosylated hemoglobin). HR: Hazard ratio; CI: Confidence interval.

Figure 2 presents multivariable-adjusted HRs for the composite endpoint according to a 1 g/dL increase in the hemoglobin levels. The adjusted HR for the highest and middle tertiles compared to the lowest tertile of hemoglobin was 0.82 (P = 0.8457) and 0.28 (P = 0.0246), respectively, in the adjusted model (demographic factors, traditional risk factors, TC, and triglycerides). However, after further adjustment for the duration of diabetes, fasting glycaemia, and HbA1c, hemoglobin was positively related to the risk of the composite endpoint (HR: 1.05, P = 0.9602) when the highest tertile was

compared to the lowest tertile of hemoglobin.

Figure 3 shows a U-shaped exposure-response relationship between admission hemoglobin levels and the composite endpoint after adjusting for age, gender, BMI, baseline eGFR, history of hypertension, SBP, DBP, 24-h urinary protein, ACEI and/or ARB use, ALB, TC, TG, duration of diabetes, fasting glycaemia, and HbA1c. The curve tended to reach the lowest level at an optimal hemoglobin level (approximately 13.3 g/dL). A higher admission hemoglobin level (hemoglobin < 13.3 g/dL) was associated with a lower risk of the composite endpoint (HR: 0.58, P = 0.0007). However, when hemoglobin was > 13.3 g/dL, the adjusted HR was 1.63 (P = 0.1585). Details are shown in Table 2.

Kaplan–Meier curves show that patients within the upper tertiles of hemoglobin had a lower cumulative incidence of the composite endpoint (Figure 4).

Stratified analysis of the composite endpoint incidence

We performed stratified analyses by age (< 60 *vs* ≥ 60 years), gender, BMI (< 24 *vs* ≥ 24 kg/m²), and ACEI/ARB use (yes *vs* no). In our cohort, the impact of hemoglobin on the composite endpoint was not affected by age, gender, BMI, or ACEI/ARB use during the follow-up period (P for all interactions > 0.05) (Figure 5).

DISCUSSION

In this retrospective observational study involving patients with T2DM, we observed the relationship between the admission hemoglobin levels and the composite endpoint. In the Cox regression model, high levels of hemoglobin were associated with a decreased risk of the composite endpoint after adjusting for age, gender, BMI, traditional risk factors, TC, and TG.

By stratifying the patients into subgroups, the results showed that the impacts of hemoglobin on the composite endpoint were not affected by age, gender, BMI, or ACEI/ARB use. Many studies also showed that low hemoglobin was associated with adverse clinical outcomes and poor life quality[11-13]. Shacham *et al*[14] analyzed 1248 patients diagnosed with ST-segment elevation myocardial infarction and demonstrated that a lower admission level of hemoglobin and anaemia were independent predictors of acute kidney injury. Dangas *et al*[15] showed that lower baseline hematocrit level was the most important independent predictor of contrast-induced nephropathy in CKD patients. An observational study included 788 patients with stages 3-5 CKD who underwent cardiac surgery and showed that preoperative anaemia was related to poor postoperative outcomes[16].

Although higher hemoglobin targets were suggested to reduce the requirement for transfusions and benefit patients' life quality[17-19], disadvantages were also been observed[7,20,21]. Several randomized controlled trials also showed that higher hemoglobin target levels were related to a higher risk of adverse outcomes[22-24]. In this study, after adjusting for the duration of diabetes, fasting glycaemia, and HbA1c, hemoglobin was positively related to the risk of the composite endpoint when the highest tertile was compared to the lowest tertile of hemoglobin. However, it is not statistically significant. We further performed a smooth spline curve and found a U-shaped exposure-response relationship between admission hemoglobin levels and the composite endpoint. The curve tended to reach the lowest level at an optimal hemoglobin level (approximately 13.3 g/dL). For the optimal hemoglobin level, lower and higher hemoglobin concentrations were related to higher rates of poor prognosis [25,26].

Furthermore, two large randomized controlled trials[24,27] showed no benefits of a higher hemoglobin target on cardiovascular events or death or found an increased rate of adverse events. Holst *et al*[28] found that patients assigned to blood transfusion at a higher hemoglobin threshold and at a lower threshold were similar on mortality at 90 d and risks of ischaemic events. Higher hemoglobin level patients had no benefit.

There are several explanations for the relationship between admission hemoglobin levels and the deterioration of renal function. Lower hemoglobin levels might decrease oxygen delivery and cause renal medullary hypoxia. The outer medullary region has high metabolic activity and low prevailing oxygen tension, so it is susceptible to ischaemic injury[29]. Angiotensin II is supposed to be a possible reason for tissue hypoxia during early CKD. The activated renin-angiotensin system induces tubular sodium reabsorption and vasoconstriction, so it results in higher oxygen consumption and relative tubular hypoxia[30-32]. However, an excessive hemoglobin level can result in increased blood viscosity and elevated blood pressure. Therefore, there is a U-

Table 2 Threshold effect analysis of hemoglobin levels on the composite endpoint using piecewise linear regression

Cutoff point of hemoglobin level (K)	Hazard ratio (95%CI)[1]	*P* value
< 13.3 g/dL	0.58 (0.42-0.79)	0.0007
≥ 13.3 g/dL	1.63 (0.83-3.20)	0.1585

[1]Adjusted for (model 4): Age, gender, body mass index, baseline estimated glomerular filtration rate, history of hypertension, systolic blood pressure, diastolic blood pressure, 24-h urinary protein, angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers use, albumin, total cholesterol, triglycerides, duration of diabetes, fasting glycaemia, and glycosylated hemoglobin.

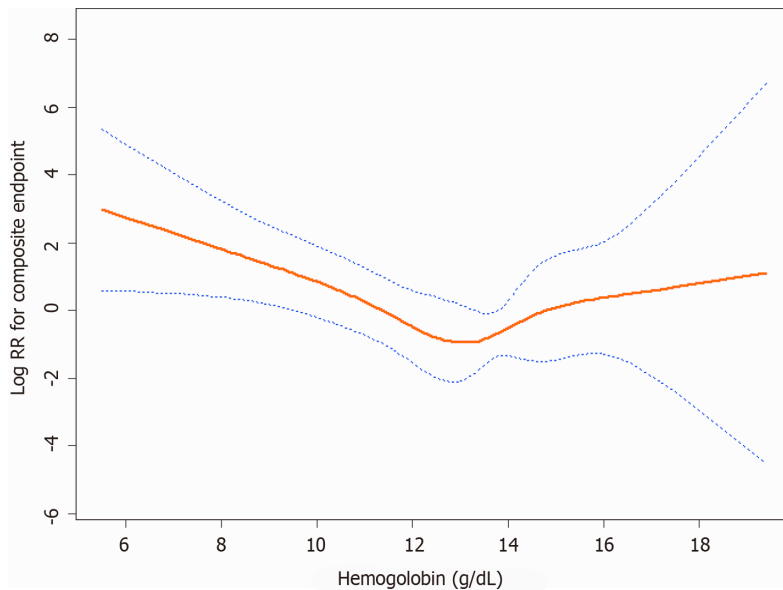A 13.3 g/dL threshold for the hemoglobin level existed for risk of the composite endpoint.

Figure 3 A U-shaped relationship was observed between hemoglobin levels and the composite endpoint. The curve tended to reach the lowest level at an optimal hemoglobin level (approximately 13.3 g/dL). The red line denotes fitted curves, and the blue lines represent 95%CI for the association between hemoglobin levels and the composite endpoint. Adjusted for (model 4): Age, gender, body mass index, baseline estimated glomerular filtration rate, history of hypertension, systolic blood pressure, diastolic blood pressure, 24 h urinary protein, angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers use, albumin, total cholesterol, triglycerides, duration of diabetes, fasting glycaemia, and glycosylated hemoglobin.

shaped exposure-response relationship between hemoglobin levels and the composite endpoint. In our study, approximately 13.3 g/dL is an optimal hemoglobin level.

We acknowledge that there are several limitations to our study. First, this was a single-center, retrospective, nonrandomized trial. It was based on observational data, and many known or unknown confounding factors may exist, even though we attempted to adjust for confounding factors. Second, we used Scr to estimate GFR. As tubular secretion of creatinine and the variability in creatinine generation between individuals and for the same individual, the use of creatinine to estimate GFR has some limitations[33]. The eGFR can be validated by serum cystatin C or 51Cr-EDTA GFR, but these tools were not available in our study. Third, there were indeed fewer outcomes among patients with a higher Hb. Thus, the CIs around the estimates for the composite outcome in Figure 3 are very wide above an Hb >13 g/dL. The reason may be that this is a small-sized study. Fourth, this was only a single-center study, and whether these observations can be extended to whole Chinese and non-Chinese T2DM patients remains to be determined.

CONCLUSION

In brief, the results of this study demonstrated that among patients with T2DM, a U-shaped exposure-response relationship exists between admission hemoglobin levels and the composite endpoint. In our study, approximately 13.3 g/dL is an optimal hemoglobin level. A lower admission hemoglobin level (hemoglobin < 13.3 g/dL) is an
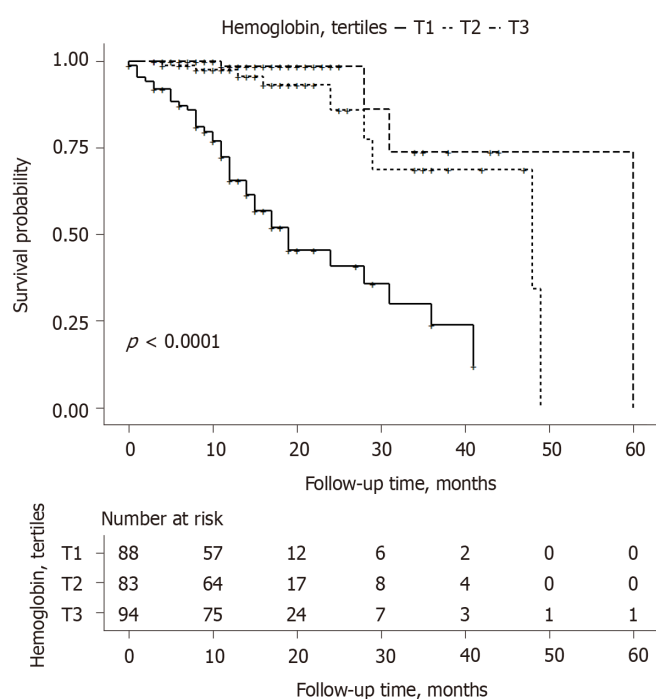
Figure 4 Kaplan–Meier curves according to hemoglobin tertiles.

outcomes	HR	95%CI	*P* for interaction
Gender			**0.3361**
male	0.71	(0.48,1.06)	
female	0.52	(0.31,0.89)	
Age, years			
<60	0.78	(0.54, 1.12)	0.3909
>=60	0.77	(0.56, 1.06)	
BMI,kg/m2			**0.4252**
<24	0.93	(0.51,1.69)	
>=24	0.7	(0.50,0.98)	
ACEI/ARB use			**0.061**
yes	0.96	(0.70,1.32)	
no	0.52	(0.32,0.84)	
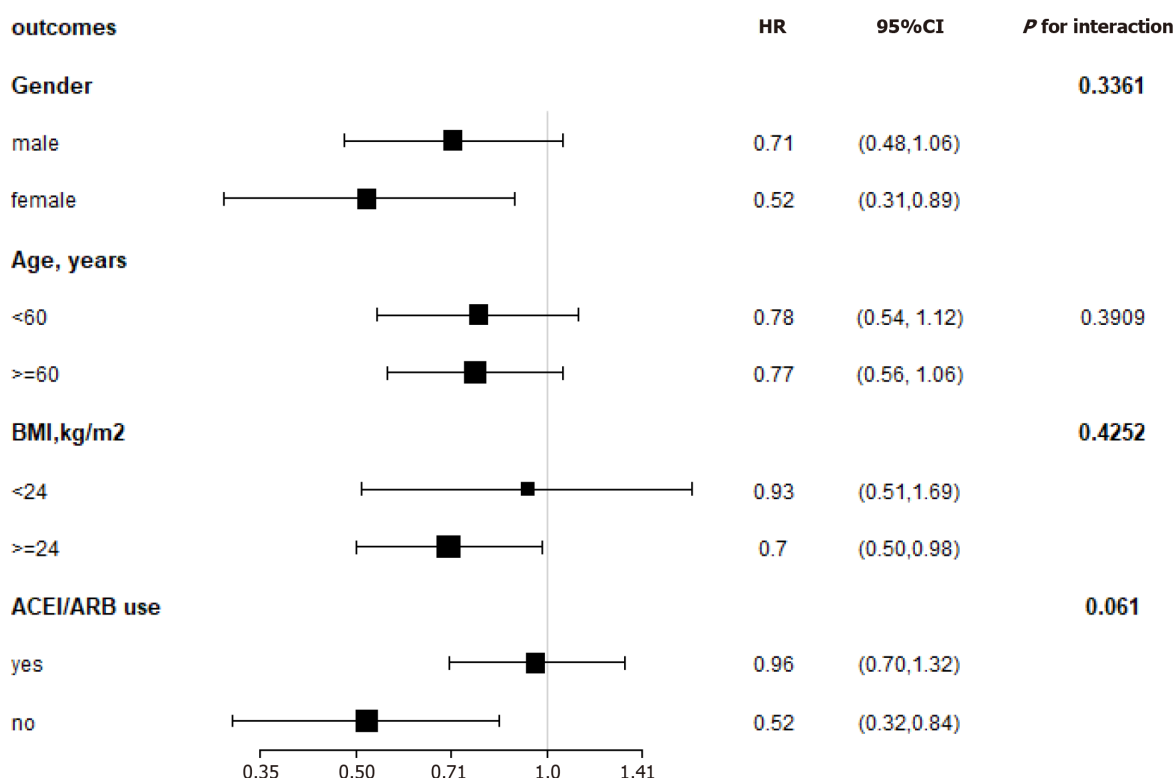
Figure 5 Subgroup analysis of hemoglobin and the composite endpoint. Adjusted for the baseline estimated glomerular filtration rate, systolic blood pressure, diastolic blood pressure, 24-h urinary protein, albumin, total cholesterol, triglycerides, duration of diabetes, fasting glycaemia, and glycosylated hemoglobin. BMI: Body mass index; ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blockers; HR: Hazard ratios.

independent predictor of the composite endpoint. These findings have important clinical and public health implications. As hemoglobin is a common and easily available measurement in clinical activity, it is a convenient and feasible way to identify those patients who are at high risk of developing the composite endpoint and have a poor prognosis.

ARTICLE HIGHLIGHTS

Research background

Information on the association of hemoglobin with renal damage is uncertain, and the optimal hemoglobin target remains controversial.

Research motivation

The admission hemoglobin levels would influence the prognosis in patients with type 2 diabetes mellitus (T2DM).

Research objectives

To evaluate the relationships between admission hemoglobin levels and prognosis in patients with T2DM

Research methods

A total of 265 patients with T2DM were included to perform a retrospective analysis. The general information and biochemical indices of these patients were statistically analyzed.

Research results

We found a U-shaped relationship between hemoglobin levels and the composite endpoint. The curve tended to reach the lowest level at an optimal hemoglobin level.

Research conclusions

There is a U-shaped relationship between hemoglobin levels and renal damage in these patients.

Research perspectives

We used a retrospective study to evaluate the relationships between admission hemoglobin levels and prognosis in patients with T2DM.

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ORIGINAL ARTICLE

Observational Study

Role of hepatitis A virus in diabetes mellitus

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Informed consent statement: All study participants, or their legal

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Abstract

BACKGROUND
Although much information is available regarding hepatitis C virus infection and diabetes, less is known about the relationship between hepatitis A virus (HAV) infection and diabetes.

AIM
To examine the roles of HAV in diabetes risk.

METHODS
This cross-sectional study population included data from the National Health and Nutrition Examination Survey collected between 2005-2012. Adult subjects (≥ 20 years old) with available body mass index measurements, defined diabetes status,

history of HAV vaccination, and HAV serology were included. HAV vaccination was based on self-reported history. Successful HAV immunization was defined as the presence of both vaccination and anti-HAV antibody. HAV infection was defined by the absence of vaccination but presence of anti-hepatitis A antibody. The odds ratio (OR) for diabetes with 95% confidence intervals (95%CI) was calculated for each HAV status and then adjusted for covariates. Sensitivity tests, based on different definitions of diabetes, were performed to verify the results.

RESULTS
Among 19942 subjects, 4229 subjects (21.21%) received HAV vaccination and HAV antibody was present in 9224 subjects (46.25%). Although HAV infection was associated with an increased risk of diabetes (OR: 1.13; 95%CI: 1.08-1.18), HAV vaccination was not associated with diabetes (OR: 1.06; 95%CI: 0.95-1.18), and successful HAV immunization had no impact on the risk of diabetes (OR: 1.11; 95%CI: 0.97-1.27). Thus, HAV infection was an unlikely cause of diabetes. Alternatively, in non-vaccinated subjects, diabetes increased the risk of HAV infection by 40% (OR: 1.40, 95%CI: 1.27-1.54).

CONCLUSION
An association between HAV infection and diabetes is observed which is best explained by an increased risk of HAV infection in diabetic patients. Diabetic subjects are more susceptible to HAV. Thus, HAV vaccination is highly recommended in diabetic patients.

Key Words: Vaccination; Immunization; Viral hepatitis; Liver; Glucose metabolism; Diabetes mellitus

Core Tip: The relationship of hepatitis A virus (HAV) infection and diabetes was examined. Although an association between HAV infection and diabetes was observed, neither HAV vaccination or successful HAV immunization had no impact on the risk of diabetes. Thus, HAV infection did not increase the risk of diabetes. In contrast, in non-vaccinated subjects, diabetes increased the risk of HAV infection by 40%. Thus, diabetic patients should receive HAV vaccination.

INTRODUCTION

Diabetes is a multifactorial disorder with both genetic and environmental components. Infection is an environmental component that plays a role in the development of insulin resistance and diabetes[1]. Although infection *per se* cannot cause diabetes, it is a well-known cause of inflammation; thus, infection may increase the risk of insulin resistance and diabetes through increased inflammation[2]. Various infectious agents have been implied in diabetes, including *helicobacter pylori*[3], hepatitis C[4,5], hepatitis B[6], and cytomegalovirus[7]. Given the relationship between hepatitis C and B with diabetes, we reasoned that a similar relationship could exist between hepatitis A virus (HAV) status and diabetes.

HAV is an enveloped RNA virus[8] that is transmitted *via* the fecal-oral route, and produces both symptomatic and asymptomatic infections[9]. Jaundice, the cardinal manifestation, develops in less than 15% of HAV-infected patients from a community outbreak[10]. HAV infection is mostly self-limited, results in life-time immunity, and does not typically result in chronic infection or chronic liver disease[11]. In the United States, HAV infection rates have declined by 95% since the HAV vaccine first became available in 1995[12]. The Centers for Disease Control and Prevention (CDC) reported

1390 acute, symptomatic cases of HAV infection in the United States in 2015, with an overall incidence rate of 0.4 cases per 100000[13]. Further, incidence may be underestimated, as HAV infection is only estimated to cause identifiable illness in less than 5% of cases[14]. As it is a food-borne disease, epidemics can be widespread and lead to significant economic loss. Thus, HAV infection remains an important public health issue.

Case reports of diabetes developed after HAV infection have led to the suggestion that HAV infection may play a role in the development of diabetes[15,16]. Based on the presence or absence of immunoglobulin G antibody to HAV, the role of HAV in diabetes was excluded in one study[17]. However, the presence of immunoglobulin G antibody to HAV could be the result of either prior HAV infection or vaccination. To address this issue, we examined the role of HAV infection, vaccination history, and successful immunization in the risk of the development diabetes in a representative United States population.

MATERIALS AND METHODS

Study population
The National Health and Nutrition Examination Survey (NHANES) is a major program of the National Center for Health Statistics (NCHS), which is part of the CDC. NHANES is designed to assess the health and nutritional status of adults and children in the United States through interviews, physical examinations, and laboratory tests. The main purpose of this survey is to provide vital and health statistics for the United States. The NCHS Research Ethics Review Board approved data collection for the NHANES 2005-2012. Informed consent was obtained from participants. The records/information were anonymized and de-identified prior to release in the NHANES website (http://www.cdc.gov/nchs/nhanes/about_nhanes.htm). Analysis of de-identified data from the survey is exempt from the federal regulations for the protection of human research participants. This study only included de-identified data from the survey.

This study used data collected during the four cycles of the NHANES survey, 2005-2006, 2007-2008, 2009-2010, and 2011-2012, as the oral glucose tolerant tests was reintroduced since 2005-2006 cycle, yielded a total of 40790 potential subjects. As questionnaires were only collected for subjects 20 years or older, 18098 subjects younger than 20 years old were excluded from this study. Body mass index (BMI) is one of the major confounding factors for the development of diabetes; thus, the 1174 subjects lacking BMI data were excluded from this study. Undefined diabetes status (based on the criteria below) excluded an additional 998 subjects. Lack of HAV vaccination status and HAV serology data, including 6 subjects with intermediate HAV antibody titer, excluded an additional 578 subjects and yielded the final study population of 19942 subjects (Figure 1).

Laboratory methods for NHANES data used in this study
Plasma glucose concentration: NHANES collected blood samples for fasting plasma glucose concentration (FPG) after a 9-h fast and 2-h plasma glucose concentration (2hPG) in 2 h after oral administration of a standardized dose (75 g) of glucose loading in a subset of subjects after obtaining fasting samples. Plasma glucose concentration was determined by a hexokinase method. To realign plasma glucose concentrations from different assays between the cycles of 2005-2006 and 2007-2012, a regress equation as recommended by NHANES was used to line up plasma glucose concentrations from 2005-2006.

Glycosylated hemoglobin: NHANES measured glycosylated hemoglobin (HbA1c) using high performance liquid chromatography-based assays. The results of HbA1c were reviewed extensively by the National Glycohemoglobin Standardization Program (NGSP) for some minor drips between cycles which could be the results of different HbA1c laboratory instruments and laboratories were used between 2005 and 2012. Since both laboratories participated in the NGSP with the recommended standardization procedures to, the NGSP concluded that both laboratories met the NGSP criteria for bias and precision and no cross-over regression was required. In this study, the re-released HbA1c data in March 2012 for 2007-2008 (GHB_E) and 2009-2010 (GHB_F) were utilized.

Hepatitis A antibody: NHANES collected human serum or plasma and performed a
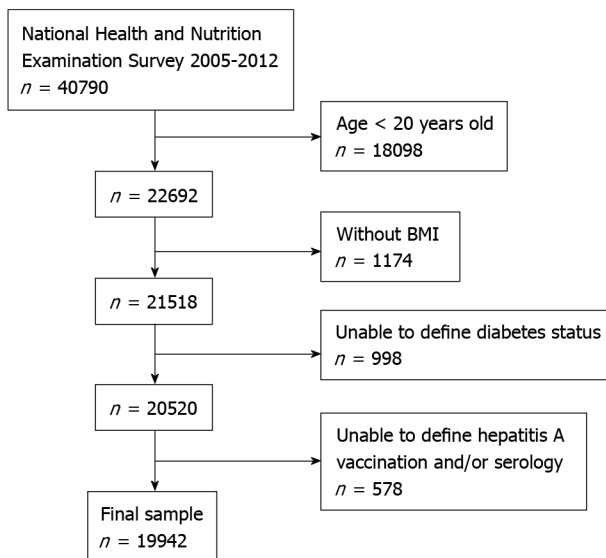
Figure 1 Sampling scheme. BMI: Body mass index.

qualitative determination of total anti-HAV antibody (both immunoglobulin G and immunoglobulin M) using competitive immunoassay. NHANES quality assurance and quality control protocols meet the 1988 Clinical Laboratory Improvement Act mandates. The assay used cannot differentiate between natural infection and vaccination; therefore, the presence of anti-HAV antibodies may reflect either acquired through infection or vaccine-induced immunity. Subjects with intermediate HAV antibody (n = 6) were excluded from the analysis.

Diabetes status
To ensure inclusion of all diabetic subjects, we defined diabetes by any of the following: (1) Subjects used diabetic medications or insulin, or were informed to be diabetic by a health care provider; 92) HbA1c ≥ 47.5 mmol/mol or 6.5%; 93) FPG ≥ 7.0 mmol/L or 126 mg/dL; or 94) 2hPG ≥ 11.1 mmol/L or 200 mg/dL, according to the American Diabetes Association[18].

Status of HAV vaccination, immunization, and infection
HAV vaccination status was self-reported to NHANES by participants. As the presence of anti-HAV antibody could be the result of prior infection or vaccination, we defined HAV infection as the presence of anti-HAV antibody in the absence of self-reported HAV vaccination. Since the successful HAV immunization rates between those who received at least 2 doses (n = 3730) or less than 2 doses (n = 499) were very similar (54.12% *vs* 52.10%, respectively, P = 0.39), they were pooled for further analysis. We defined successful HAV immunization as the presence of both anti-HAV antibody and HAV vaccination history.

Other covariate measures: NHANES-collected data on continuous covariates including age, BMI, and poverty index, and categorical covariates including gender, race/ethnicity, smoking status, alcohol consumption status, immediate family of diabetes, and education levels, were considered in this study. Age was calculated in years at the time of the screening interview form the reported date of birth. BMI (kg/m²) was computed from measured weight (kg) divided by the square of measured standing height (m). Based on the poverty guidelines of the United States Department of Health and Human Services, poverty index was computed as the ratio of family income to poverty, as described previously[19]. The reference poverty index of 1.0 denotes family income at the federal poverty level, and a poverty index of 2.0 signifies family income that is 200% of the federal poverty level. Gender was based on self-reported categories from the participants. Similarity, based on self-reported racial/ethnic groups, ethnicity/race were further categorized as Mexican Americans, other Hispanics, non-Hispanics whites, non-Hispanic blacks, and other ethnic/racial groups. Smoking status was defined as using tobacco/nicotine in the last 5 d. Status of alcohol consumption was defined as current alcohol consumption based on at least 12 alcohol drinks per year in the past year. Based on any blood relatives, including father,

mother, sisters or brothers, ever being told by a health professional that they had diabetes, family history of diabetes was defined as positive. Based on the self-reported highest education, education level was categorized into 2 major categories: less or at least high school graduate.

Statistical analysis

SYSTAT 13.0 for windows package from SPSS, INC. (Chicago, IL, United States) was used for statistical analysis. Continuous data were presented as mean ± SD and examined using a two-tailed Student *t*-test or ANOVA test as if appropriate. Differences in categorical data were expressed in proportions and Chi-square test was used to examine categorical data. The odds ratio (OR) with 95% confidence intervals (CI) of risk of diabetes based on HAV vaccination history, HAV infection, and successful HAV immunization, or risk of HAV infection based on diabetes, were calculated based on logistic regression analyses with the contemplation of covariates (described above). No covariate was considered in Model 1; gender, age, and BMI were considered in Model 2; ethnic/racial group, gender, age, and BMI were considered in Model 3; all covariates including gender, age, BMI, race/ethnicity, current smoker, current alcohol consumption, family history of diabetes, poverty index, and education level were considered in Model 4. A nominal *P* value < 0.05 was considered to be significant.

RESULTS

Study population

To study the relationship between HAV and diabetes, we collected relevant data from NHANES. The sampling scheme was shown in Figure 1 and the clinical features of the study population (*n* = 19,942) were shown in Table 1. The prevalence of diabetes was 17.37%, with 12.19% established and 5.18% undiagnosed diabetic subjects. We used the distribution of anti-HAV antibody and vaccination history to calculate HAV immunization and infections rates (Table 2). Anti-HAV antibody was present in 9224 subjects (46.25% of total subjects), and 4229 subjects (21.21% of total subjects) received HAV vaccination (Table 2). We defined successful immunization as the intersection of the presence of anti-HAV antibody and HAV vaccination history; successful HAV immunization occurred in 2279 subjects (53.89% of vaccinated subjects). We defined HAV infection as the intersection of the presence of anti-HAV antibody with no history of HAV vaccination; infection occurred in 6945 subjects (44.20% of non-vaccinated subjects).

Viral hepatitis A infection and diabetes

To evaluate the role of HAV infection in the risk of development of diabetes, we compared the prevalence of diabetes between two groups: HAV infected subjects (the presence of anti-HAV antibody without a history of HAV vaccination) *vs* non-HAV infected subjects. As shown in Table 3, diabetes occurred in 1712 subjects (24.65%) with HAV infection and in 1751 subjects (13.47%) without HAV infection (*P* < 0.0001), with an OR of 1.45 (95%CI: 1.40-1.50; Table 4). Although no difference was noted in the gender distribution (*P* = 0.11) or BMI (*P* = 0.07), HAV-infected subjects were significantly older than non-infected subjects (56 ± 18 years and 46 ± 17 years, respectively; *P* < 0.0001). After adjustment for gender, age, and BMI covariates, HAV infection was significantly associated with an increased OR for diabetes (1.22; 95%CI: 1.17-1.27; Table 4). Although the frequency of HAV infection varied significantly across ethnic/racial groups (*P* < 0.0001), additional adjustment with ethnic/racial group had no significant effect on diabetes risk when considered as a covariate with gender, age, and BMI (OR: 1.22; 95%CI: 1.17-1.28; Table 4). In the fully adjusted Model 4 that includes all covariates, HAV infection remained a significant risk factor for the development of diabetes (OR: 1.13; 95%CI: 1.08-1.18; Table 4). Thus, there was an association between HAV infection diabetes.

To further evaluate the association of HAV with diabetes, we conducted sensitivity analyses with various definitions of diabetes (Table 4). As established diabetes accounted for 70.17% of diabetes in this sample set, we first examined the role of HAV infection in established diabetes. The results of all four models confirmed an association between HAV infection and established diabetes (Table 4). Using FPG and 2hPG concentrations as a proxy for diabetes confirmed the association between HAV infection and diabetes, demonstrating similar ORs to established diabetes for all four

Table 1 Study population clinical characteristics, *n* (%)

n		19942
Gender	Female	10256 (51.43)
Age	Year	49 ± 18
Body mass index	Kg/m²	28.94 ± 6.75
Fasting plasma glucose[1]	mmol/L	6.1 ± 2.1
2-h plasma glucose[2]	mmol//L	6.8 ± 2.9
HbA1c[3]	%	5.7 ± 1.0
HbA1c[3]	mmol/mol	38.90 ± 11.45
Current smoker	Yes	4651 (23.32)
Current alcohol consumption	Yes	13069 (65.54)
Education: at least high school graduate	Yes	14467 (72.55)
Poverty index		2.72 ± 1.70
Family history of diabetes	Yes	7936 (39.80)
States of glucose tolerance		
Diabetes	Yes	3463 (17.37)
Established diabetes	Yes	2430 (12.19)
Undiagnosed diabetes	Yes	1033 (5.18)
Hepatitis A vaccination	Yes	4229 (21.21)
Presence of anti-HAV antibody	Yes	9224 (46.25)
Ethnic/racial groups		
Mexican American		3307 (16.58)
Other Hispanic		1787 (8.96)
Non-Hispanic white		9205 (46.16)
Non-Hispanic black		4131 (20.72)
Other		1512 (7.58)

[1]*n* = 7105.
[2]*n* = 7125.
[3]*n* = 19910.
Mean ± STD or *n* percent.
HAV: Hepatitis A virus.

Table 2 Distribution of hepatitis A antibody and vaccination history, *n* (%)

	No for HAV vaccination	Yes for HAV vaccination	Subtotal[1]
Anti-HAV antibody presence	6945 (44.20)	2279 (53.89)	9224 (46.25)
Anti-HAV antibody absence	8768 (55.80)	1950 (46.11)	10718 (53.75)
Subtotal[1]	15713 (78.79)	4229 (21.21)	19942 (100.00)

[1]*n* (raw percent).
Pearson Chi-Square = 125.876927, degree of freedom = 1, *P* < 0.0001. *n* (column percent). HAV: Hepatitis A virus.

models (Table 4, respectively). As only a selected subset of subjects had either FPG or 2hPG measurements, the sample size was reduced significantly, but P-values, although increased, remained significant (*P* = 0.01 and *P* = 0.04, respectively). As HbA1c measurements were available for most subjects, the sample size, ORs, and P-values were very similar for the association of hepatitis A and HbA1c as for

Table 3 Comparison of clinic characteristics of subjects with and without hepatitis A virus infection, *n* (%)				
		Non-infected	**Infected**	***P***
n		12997	6945	
Gender	Female	6738 (51.84)	3518 (50.66)	NS
Age	Yr	46 ± 17	56 ± 18	< 0.0001
Body mass index	Kg/m^2	29.00 ± 7.01	28.82 ± 6.24	NS
Fasting plasma glucose[1]	mmol/L	5.8 ± 1.8	6.4 ± 2.4	< 0.0001
2-h postchallenged plasma glucose[2]	mmol/L	6.4 ± 2.7	7.4 ± 3.4	< 0.0001
HbA1c[3]	%	5.6 ± 0.9	5.9 ± 1.2	< 0.0001
HbA1c[3]	mmol/mol	37.66 ± 10.20	41.22 ± 13.18	< 0.0001
Current smoker	Yes	3418 (26.30)	1233 (17.75)	< 0.0001
Current alcohol consumption	Yes	9089 (69.93)	3980 (57.31)	< 0.0001
Education: at least high school graduate	Yes	10668 (82.08)	3799 (54.70)	< 0.0001
Poverty index		2.87 ± 1.70	2.44 ± 1.66	< 0.0001
Family history of diabetes	Yes	5074 (39.04)	2862 (41.21)	0.003
Established diabetes	Yes	1217 (9.36)	1213 (17.47)	< 0.0001
States of glucose tolerance				< 0.0001
Normal glucose tolerance		7512 (57.80)	2799 (40.30)	
Impaired glucose tolerance		3734 (28.73)	2434 (35.05)	
Diabetes		1751 (13.47)	1712 (24.65)	
Hepatitis A vaccination	Yes	4229 (32.54)	0 (0.00)	< 0.0001
Presence of hepatitis A antibody	Yes	2279 (17.53)	6945 (100.00)	< 0.0001
Ethnic/racial				< 0.0001
Mexican American		1184 (9.11)	2123 (30.57)	
Other Hispanic		800 (6.16)	987 (14.21)	
Non-Hispanic white		7346 (56.52)	1859 (26.77)	
Non-Hispanic black		2807 (21.60)	1,324 (19.06)	
Other		860 (6.62)	652 (9.39)	

Mean ± STD.

[1]For non-infected: *n* = 4579; For infected: *n* = 2526.

[2]For non-infected: *n* = 4777; For infected: *n* = 2438.

[3]For non-infected: *n* = 12978; For infected: *n* = 6932.

established diabetes (Table 4).

HAV vaccination and diabetes

Since HAV infection was associated with an increased risk for diabetes, we next examined whether HAV vaccination was associated with a reduced risk of diabetes (Table 5). In this population, 12.84% of subjects who received HAV vaccination were diabetic, while 18.58% of non-vaccinated subjects were diabetic (*P* < 0.0001). The clinical characteristics of the two groups also differed significantly, except for the status of smoking and alcohol consumption. However, the association disappeared after adjustment for age, gender, and BMI in Model 2, and further analyses (Models 3 and 4) excluded the role of HAV vaccination in diabetes (Table 5). In the sensitivity analyses, the protective effect of diabetes by HAV vaccination was noted in Model 1 (unadjusted) by all diabetes definitions (Table 5), but adjustment of covariates (Models 2, 3, and 4) rendered no association between HAV vaccination and diabetes. Thus, HAV vaccination had no impact on the development of diabetes.

Table 4 Odds ratio and 95% confidence intervals for diabetes by hepatitis A virus infection, *n* (%)

Status	HAV infection	*n*	Diabetes	Model 1	Model 2	Model 3	Model 4
All diabetes	No	12997	1751 (13.47)	1.45	1.22	1.22	1.13
	Yes	6945	1712 (24.65)	(1.40-1.50)	(1.17-1.27)	(1.17-1.28)	(1.08-1.18)
Established diabetes	No	12997	1217 (9.36)	1.43	1.20	1.21	1.11
	Yes	6945	1213 (17.47)	(1.37-1.49)	(1.14-1.26)	(1.15-1.27)	(1.05-1.16)
Diabetes by fasting plasma glucose criterion	No	4579	432 (9.43)	1.44	1.22	1.21	1.12
	Yes	2526	451 (17.85)	(1.35-1.55)	(1.13-1.32)	(1.12-1.31)	(1.03-1.21)
Diabetes by 2-h postchallenged plasma glucose criterion	No	4777	277 (5.80)	1.46	1.21	1.17	1.11
	Yes	2348	274 (11.67)	(1.34-1.60)	(1.10-1.33)	(1.07-1.29)	(1.01-1.23)
Diabetes by HbA1c criterion	No	12978	1023 (7.88)	1.47	1.27	1.28	1.19
	Yes	6932	1085 (15.65)	(1.41-1.54)	(1.21-1.34)	(1.22-1.35)	(1.12-1.25)

Model 1, unadjusted; Model 2, adjusted for gender, age, and body mass index (BMI); Model 3, adjusted for gender, age, BMI, and ethnic/racial group; Model 4, adjusted for gender, age, BMI, ethnic/race group, active smoker, active alcohol consumption, family history of diabetes, poverty index, and education. HAV: Hepatitis A virus.

Table 5 Odds ratio and 95% confidence intervals for diabetes by hepatitis A virus vaccination, *n* (%)

Status	HAV vaccination	*n*	Diabetes	Model 1	Model 2	Model 3	Model 4
All diabetes	No	15713	2920 (18.58)	0.65	1.02	1.03	1.06
	Yes	4229	543 (12.84)	(0.58-0.71)	(0.91-1.14)	(0.92-1.14)	(0.95-1.18)
Established diabetes	No	15713	2039 (12.98)	0.68	1.08	1.08	1.12
	Yes	4229	391 (9.25)	(0.61-0.77)	(0.96-1.22)	(0.96-1.22)	(0.99-1.28)
Diabetes by fasting plasma glucose criterion	No	5578	750 (13.45)	0.61	0.95	0.95	0.97
	Yes	1527	133 (8.71)	(0.51-0.75)	(0.77-1.16)	(0.77-1.17)	(0.78-1.19)
Diabetes by 2-h postchallenged plasma glucose criterion	No	5550	478 (8.61)	0.52	0.85	0.86	0.88
	Yes	1575	73 (4.63)	(0.40-0.66)	(0.65-1.10)	(0.66-1.12)	(0.67-1.15)
Diabetes by HbA1c criterion	No	15690	1772 (11.29)	0.68	1.03	1.03	1.06
	Yes	4220	336 (7.96)	(0.60-0.77)	(0.90-1.17)	(0.90-1.17)	(0.93-1.21)

Model 1, unadjusted; Model 2, adjusted for gender, age, and body mass index (BMI); Model 3, adjusted for gender, age, BMI, and ethnic/racial group; Model 4, adjusted for gender, age, BMI, ethnic/race group, active smoker, active alcohol consumption, family history of diabetes, poverty index, and education. HAV: Hepatitis A virus.

Successful HAV immunization and diabetes

Since we noted the presence of anti-HAV antibody in only 53.89% of vaccinated subjects (Table 2), HAV vaccination per se might not be effective at all in the reduction of diabetes. We examined the role of successful HAV immunization on risk of diabetes, defined as the presence of both HAV vaccination and anti-HAV antibody. The prevalence of diabetes was significantly different between the subjects with and without successful immunization (14.79% *vs* 17.70%, respectively, *P* = 0.0006). Clinical characteristics also differed significantly between the two groups, except for education. As shown in Table 6, although an unadjusted analysis (Model 1) demonstrated a protective effect, additional analyses in Models 2, 3, and 4 failed to confirm the protective effect of successful HAV immunization on the risk of diabetes. Similar results were obtained in the sensitivity analyses (Table 6). Thus, successful HAV immunization played no role in the development of diabetes.

Viral hepatitis A infection and diabetes in non-vaccinated subjects

To examine the alternative hypothesis that diabetes was more susceptible for HAV

Table 6 Odds ratio and 95% confidence intervals for diabetes by successful hepatitis A virus immunization, *n* (%)

Status	Successful HAV immunization	*n*	Diabetes	Model 1	Model 2	Model 3	Model 4
All diabetes	No	17663	3126 (17.70)	0.81	1.10	1.10	1.11
	Yes	2279	337 (14.79)	(0.71-0.91)	(0.96-1.26)	(0.96-1.25)	(0.97-1.27)
Established diabetes	No	17512	2039 (11.64)	0.83	1.13	1.13	1.14
	Yes	2430	240 (9.88)	(0.72-0.96)	(0.97-1.31)	(0.97-1.31)	(0.98-1.33)
Diabetes by fasting plasma glucose criterion	No	6244	796 (12.75)	0.77	1.06	1.05	1.05
	Yes	861	87 (10.10)	(0.61-0.97)	(0.83-1.36)	(0.82-1.35)	(0.82-1.36)
Diabetes by 2-h postchallenged plasma glucose criterion	No	6243	498 (7.98)	0.74	1.02	1.01	1.03
	Yes	882	53 (6.01)	(0.55-0.99)	(0.75-1.39)	(0.74-1.37)	(0.76-1.40)
Diabetes by HbA1c criterion	No	17636	1900 (10.77)	0.83	1.11	1.11	1.12
	Yes	2274	208 (9.15)	(0.72-0.97)	(0.95-1.30)	1.30)	(0.95-1.32)

Model 1, unadjusted; Model 2, adjusted for gender, age, and body mass index (BMI); Model 3, adjusted for gender, age, BMI, and ethnic/racial group; Model 4, adjusted for gender, age, BMI, ethnic/race group, active smoker, active alcohol consumption, family history of diabetes, poverty index, and education. HAV: Hepatitis A virus.

infection, we restricted the sample to non-vaccinated subjects only and examined the association between diabetes status and HAV infection. In non-vaccinated subjects, 58.63% of diabetic subjects were seropositive for anti-HAV antibody, while only 40.91% of non-diabetic subjects were seropositive ($P < 0.0001$). The OR for HAV infection was 2.05 (95%CI: 1.89-2.22) for Model 1, which was unadjusted for covariates (Table 7). The association remained highly significant ($P < 0.0001$ for all models) after adjustment for age, gender, and BMI in Model 2 (OR: 1.59, 95%CI: 1.45-1.74), additional ethnic/racial groups in Model 3 (OR: 1.65, 95%CI: 1.50-1.81), and all covariates in Model 4 (OR: 1.40, 95%CI: 1.27-1.54). Sensitivity analyses with various definition of diabetes confirmed an increased OR for HAV infection in diabetic subjects (Table 7). Thus, in non-vaccinated subjects, subjects with diabetes had an increased risk of HAV infection than subjects without diabetes.

DISCUSSION

In this study, we examined the relationship between HAV status and diabetes. The primary hypothesis was that HAV infection increases the risk of diabetes, while alternatively diabetes increased susceptibility to HAV infection, given another possibility would be no association between HAV infection and diabetes. We performed a stepwise hypothesis testing approach. First, the association of HAV infection with diabetes was established, followed by examination of the effect of HAV vaccination on diabetes. After demonstrating a lack of association with HAV vaccination, we further examined the effect of successful HAV immunization on diabetes, but again found no association. Instead, we confirmed an alternative hypothesis that diabetes increased susceptibility to HAV infection.

To examine the hypothesis that HAV was a risk factor for diabetes, we first examined the association between HAV infection and diabetes. In this representative U.S. population, the prevalence of HAV infection is 34.83% and HAV infection is associated with a 13% increased risk of diabetes (OR: 1.13, 95CI%: 1.08-1.18) after adjustment of covariates (Table 4). In contrast to viral hepatitis B and C, HAV infection is not associated with chronic liver disease[8,9,11]. The possibility of previous HAV infection affecting glucose metabolism could not be excluded. From a metabolic point of view, the HAV-infected subjects had significantly higher FPG, 2hPG, and HbA1c than non-HAV-infected subjects (Table 3). These results further suggest the possible interaction between a previously HAV-infected liver and glucose metabolism. Nevertheless, our results demonstrate a link between the serological evidence of HAV infection and diabetes initially.

Table 7 Odds ratio and 95% confidence intervals for hepatis A virus infection by diabetes status in non-hepatitis A vaccinated participants, *n* (%)

Status	Diabetes	*n*	HAV infection	Model 1	Model 2	Model 3	Model 4
All diabetes	No	12793	5233 (40.91)	2.05	1.59	1.65	1.40
	Yes	2920	1712 (58.63)	(1.89-2.22)	(1.45-1.74)	(1.50-1.81)	(1.27-1.54)
Established diabetes	No	13674	5732 (41.92)	2.03	1.57	1.67	1.39
	Yes	2039	1213 (59.49)	(1.85-2.24)	(1.42-1.73)	(1.50-1.85)	(1.24-1.56)
Diabetes by fasting plasma glucose criterion	No	4828	2075 (42.98)	2	1.56	1.58	1.34
	Yes	750	451 (60.13)	(1.71-2.34)	(1.32-1.85)	(1.32-1.87)	(1.11-1.61)
Diabetes by 2-h postchallenged plasma glucose criterion	No	5072	2074 (40.89)	1.94	1.48	1.39	1.27
	Yes	478	274 (57.32)	(1.61-2.35)	(1.21-1.81)	(1.13-1.71)	(1.02-1.58)
Diabetes by HbA1c criterion	No	13918	5847 (42.01)	2.18	1.76	1.87	1.59
	Yes	1772	1085 (61.23)	(1.97-2.41)	(1.58-1.96)	(1.67-2.09)	(1.42-1.80)

Model 1, unadjusted; Model 2, adjusted for gender, age, and body mass index (BMI); Model 3, adjusted for gender, age, BMI, and ethnic/racial group; Model 4, adjusted for gender, age, BMI, ethnic/race group, active smoker, active alcohol consumption, family history of diabetes, poverty index, and education. HAV: Hepatitis A virus.

If this observation was true, the HAV vaccination would reduce the risk of diabetes. Furthermore, we observed that the subjects with HAV infection history were less likely to be high school graduate or higher (54.70% *vs* 82.08%, *P* < 0.0001) than those without evidence of HAV infection. As it is well known that health-conscious persons are more likely to receive HAV vaccination, and less likely to develop diabetes, we next examined whether there was any association of HAV vaccination with diabetes, especially with reduced risk of diabetes. In this population, the HAV vaccination rate was relatively low, only 21.21%, and HAV vaccination had no impact of diabetes risk (OR: 1.06, 95%CI: 0.95-1.18) after adjustment for covariates (Table 5). Although an initial protective effect was noted in Model 1 (Table 5), no association between HAV vaccination and diabetes was found after adjustment for covariates. These results also suggested that the observed increased risk of diabetes with HAV infection was less likely through a selective process of a less health-conscious population (*i.e.*, no HAV vaccination). However, this observation contradicted the initial observed association between HAV infection and diabetes. Next, we studied the effectiveness of HAV vaccination in the study population, which could affect the association between vaccination and diabetes. HAV vaccination has been reported to be very effective[20], but we found no evidence of reduced diabetes risk with HAV vaccination (Table 5). We examined the effectiveness of HAV vaccination in this population and found only 53.89% of vaccinated subjects were noted to have adequate anti-HAV antibody (Table 2). Among 4229 subjects who received vaccination, 543 subjects were diabetic. Anti-HAV antibody was present in 62.06% of diabetic subjects who received vaccination while in 52.69% of non-diabetic subjects who received vaccination (*P* < 0.0001). This suggested that either: 1) subjects with diabetes respond to HAV vaccination better than subjects without diabetes, or 2) subjects with diabetes were more likely to have received HAV vaccination than non-diabetic subjects. Due to relatively low seropositivity for HAV after vaccination, we further examined the effect of successful HAV immunization on diabetes risk. Again, no association was found between successful HAV immunization and diabetes (Table 6). If HAV infection was a risk factor of the development of diabetes as the observed association between diabetes and HAV infection, one would expect that successful immunization of HAV would reduce diabetes risk. However, the results demonstrated that successful HAV immunization had no impact on the risk of diabetes.

As this is a cross-sectional association study, the causal relationship cannot be obtained directly from this study. With the stepwise hypothesis testing approach, the original hypothesis was less likely to be true. To reconcile the results of diabetes with HAV infection, vaccination and successful immunization, alternative hypothesis that diabetic subjects was more prone for HAV infection was investigated in non-vaccinated subjects. One would expect to see more HAV infection in diabetic subjects than non-diabetic subjects without vaccination. To exclude the effect of HAV

vaccination, the subset of non-vaccination subjects (*n* = 15713) was further examined for the association between HAV infection and diabetes (Table 7). Among 2920 diabetic subjects without HAV vaccination, the seropositive rate for anti-HAV antibody was 58.63%, while it was 40.91% in 12793 non-diabetic subjects without HAV vaccination (*P* < 0.0001). Thus, diabetes increased the risk of HAV with an OR of 2.05 (95%CI: 1.89-2.11). The association was confirmed after adjustment for covariates (Table 7) and also by sensitivity analyses based on the different definition of diabetes (Table 7). Thus, the results of this study are most consistent with the alternative hypothesis that diabetes increases the risk of HAV infection.

There are several features of this study that worthwhile to be mentioned. A representative United States population with fairly large sample size was used in the present study which makes the results more applicable to the United States population. As it is almost impossible to match the studied groups completely, the large sample size with fairly extensive data available allows adjustment of covariates properly. Indeed, adjustment of covariates did affect the results of HAV vaccination and successful HAV immunization, as protective effects were noted initially and they disappear after adjustment of covariates. In contrast, adjustment of covariates confirmed the results for the association between HAV infection and diabetes in the whole sample set as well as in the subset of subjects without prior HAV vaccination. Furthermore, we took extra steps to validate the result by sensitivity tests with different definition of diabetes to confirm the observations and the results of sensitivity tests were consistent with the original observations. Stepwise hypothesis testing approach allows this study to provide the insight of casual relationship between HAV infection and diabetes. Thus, there is a significant scientific merit of this study.

As this is a cross-sectional study, no causal relationship can be provided directly. However, with stepwise hypothesis testing approach, we are able to deduct the results and to infer diabetes as the cause of the increased susceptibility of HAV infection. Alternatively, one can examine the effect of aggressive prevention of diabetes on the incidence of HAV infection. Given the established benefit of diabetes prevention[21], it would not be ethical to conduct an interventional trial to examine the impact of diabetes prevention on the development of HAV infection. Thus, stepwise hypothesis testing approach is a useful alternative approach to infer the causal relationship when an interventional trial is not feasible to confirm the observation.

In the NHANES, there was no information collected regarding the type of diabetes and auto-antibodies for type 1 diabetes were not assessed. Thus, patients with type 1 diabetes cannot be separated out unambiguously. Based on the National Health Interview Survey in 2016, type 2 diabetes accounted for 90.9% of diabetes while type 1 diabetes accounted for 5.8% of diabetes[22] which was defined based on self-report type and also current use of insulin. In this Survey, the reported prevalence of type 1 diabetes was 0.55% of the adult population in the United Stated[22], which is much higher than the reported 0.18% from the Hispanic Community Health Study/Study of Latinos, which is a community-based epidemiologic study in Hispanic/Latino adults residing in four United States communities[23]. The overestimation in the 2016 Survey [22] could be from the assumption of type 1 diabetes based on the current use of insulin. Since type 2 diabetes develops when beta cell function to 55% left and beta cell declines at 5% per year regardless treatment modalities[24], it is expected that insulin treatment will be required sometime in the course of type 2 diabetes and it has been demonstrated that almost two-thirds of patient with type 2 diabetes received insulin treatment[25]. Thus, defining type 1 diabetes based on using insulin treatment is unreliable and could overestimate the prevalence of type 1 diabetes. Since type 1 and type 2 diabetes are the two most common form of diabetes and less than 10% of patients with diabetes have type 1 diabetes, the vast majority (≥ 90%) of diabetic patients in this cohort have type 2 diabetes. Thus, we are able to conclude the association of HAV infection with type 2 diabetes.

Hyperglycemia is associated with the increased risk of infection[26]. Infectious burden increases by 2.14 times in diabetes[27]. Furthermore, hospitalization rates from infection is almost 3.8-time higher in adults with diabetes than without diabetes, especially young adults with diabetes[28] who are more prevalent with type 1 diabetes. Thus, patients with type 1 diabetes could also have an increased risk of HAV infection. However, due to limited number of type 1 diabetes and unable to separate out type 1 diabetes unequivocally based the study design of the NHANES, we are not able to generalize the observed results in type 1 diabetes. Nevertheless, our observation of an increased risk of HAV infection in diabetes is in line with reported increased risk of infection in diabetes, regardless type of diabetes. Furthermore, the effectiveness of HAV vaccination is relatively low, 53.89% (Table 2), which is not in line with the published reports[20]. Further studies are required to address these

issues.

CONCLUSION

In summary, we have demonstrated an association between diabetes and HAV infection. This association is not mediated through acute HAV infection, and HAV vaccination and successful HAV immunization do not reduce the risk of diabetes. In non-HAV-vaccinated subjects, diabetes is a risk factor for HAV infection with an OR of 1.40 (95%CI: 1.27-1.54). Thus, through the deduction process of a stepwise hypothesis testing approach, we suggest that the most rational explanation is that diabetes increases the risk of HAV infection by 40%. Furthermore, diabetes increases HAV mortality by 2.2 times[29]. Accordingly, HAV vaccination is highly recommended in diabetic subjects.

ARTICLE HIGHLIGHTS

Research background

The liver plays an important role in glucose hemostasis. Hepatitis C infection increases the risk of diabetes. Thus, other liver infection could also play a role in the pathogenesis of diabetes.

Research motivation

This study is to dissect the relationship between hepatitis A and diabetes.

Research objectives

The objective of this study is to investigate the interaction between hepatitis A and diabetes through hypothesis testing in a representative adult cohort of the United States.

Research methods

The information on hepatitis A vaccination history, hepatitis A antibody status, and diabetes status were obtained from the participants in the National Health and Nutrition Examination Survey 2005-2012. Hepatitis A infection was defined as the presence of hepatitis A antibody without hepatitis A vaccination. Hepatitis A vaccination was defined as the presence of hepatitis A vaccination, regardless hepatitis A antibody status. Successful hepatitis A immunization was defined as the presence of both hepatitis A vaccination and hepatitis A antibody. A stepwise hypothesis testing approach was used to dissect the interaction between hepatitis A and diabetes and the sensitivity tests based on the different definitions of diabetes were used to confirm the results.

Research results

Diabetes risk was increased in the participants with hepatitis A infection, suggesting a causal relationship between hepatitis A infection and diabetes. However, hepatitis A vaccination and also successful hepatitis A immunization did not reduce the risk of diabetes, excluding hepatitis A infection as a cause of diabetes. In non-hepatitis A vaccinated participants, diabetes increased the risk of hepatitis A infection by 40%.

Research conclusions

Hepatitis A is not a cause of diabetes. Instead, diabetes increases the risk of hepatitis A infection.

Research perspectives

Since hepatitis A mortality increases in patients with diabetes, hepatitis A vaccination is highly recommended in patients with diabetes.

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Observational Study

Adherence to Mediterranean diet and advanced glycation endproducts in patients with diabetes

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Abstract

BACKGROUND
In recent years, American Diabetes Association started to strongly advocate the
Mediterranean diet (MD) over other diets in patients with diabetes mellitus (DM)
because of its beneficial effects on glycemic control and cardiovascular (CV) risk
factors. Tissue levels of advanced glycation endproducts (AGEs) emerged as an
indicator of CV risk in DM. Skin biopsy being invasive, the use of AGE Reader
has been shown to reflect tissue AGEs reliably.

AIM
To examine the association between adherence to MD and AGEs in patients with
DM type II.

METHODS
This cross-sectional study was conducted on 273 patients with DM type II. A
survey questionnaire was composed of 3 separate sections. The first part of the
questionnaire included general data and the habits of the participants. The second
part aimed to assess the basic parameters of participants' diseases and associated
conditions. The third part of the questionnaire was the Croatian version of the 14-
item MD service score (MDSS). AGEs levels and associated CV risk were
measured using AGE Reader (DiagnOptics Technologies BV, Groningen, The
Netherlands).

RESULTS

A total of 27 (9.9%) patients fulfilled criteria for adherence to MD, with a median score of 8.0 (6.0-10.0). Patients with none/limited CV risk had significantly higher percentage of MD adherence in comparison to patients with increased/definite CV risk (15.2% *vs* 6.9%, $P = 0.028$), as well as better adherence to guidelines for nuts (23.2% *vs* 12.6%, $P = 0.023$) and legumes (40.4% *vs* 25.9%, $P = 0.013$) consumption. Higher number of patients with glycated hemoglobin (HbA1c) < 7% adhered to MD when compared to patients with HbA1c > 7% (14.9% *vs* 7.3%, $P = 0.045$). Moreover, those patients followed the MDSS guidelines for eggs (33.0% *vs* 46.8%, $P = 0.025$) and wine (15.6% *vs* 29.8%, $P = 0.006$) consumption more frequently. MDSS score had significant positive correlation with disease duration ($r = 0.179$, $P = 0.003$) and negative correlation with body mass index (BMI) values ($r = -0.159$, $P = 0.008$). In the multiple linear regression model, BMI (β ± SE, -0.09 ± 0.04, $P = 0.037$) and disease duration (β ± SE, 0.07 ± 0.02, $P < 0.001$) remained significant independent correlates of the MDSS score. Patients with HbA1c > 7% think that educational programs on nutrition would be useful for patients in significantly more cases than patients with HbA1c < 7% (98.9% *vs* 92.6%, $P = 0.009$).

CONCLUSION

Although adherence to MD was very low among people with diabetes, we demonstrated that adherence to MD is greater in patients with lower CV risk, longer disease duration, and well-controlled glycaemia.

Key Words: Mediterranean diet; Cardiovascular disease; Diabetes mellitus; Advanced glycation endproducts; Dietary habits; Atherosclerosis

Core Tip: Recently, American Diabetes Association started to advocate the use of the Mediterranean diet (MD) over other diets because of its beneficial effects on glycaemic control and cardiovascular (CV) risk factors. In this cross-sectional study, we demonstrated an association between adherence to the MD and CV risk in patients with diabetes mellitus (DM) type II by measuring advanced glycation endproducts. In addition, we found that adherence to MD is very low in diabetics, especially among individuals with poorly controlled glycaemia. Finally, the duration of DM independently predicted better adherence to MD, whereas body mass index predicted poorer adherence.

INTRODUCTION

Diabetes mellitus (DM) is a group of chronic disorders of carbohydrate, fat and protein metabolism caused by defects of insulin secretion, action, or both[1]. Among two of the most prominent types, DM type II accounts for 90%-95% of people with DM[2]. Around 462 million individuals worldwide are affected by DM type II, corresponding to 6.28% of the world's population[3]. The rising incidence is even more staggering, as recent estimates point that 1 in 3 people in the world will have DM type II by 2050[4]. Even though immense efforts in terms of public health measures were made, developed regions, such as Western Europe and the United States, show considerably higher prevalence rates of DM in contrast to Third World countries[4]. This highlights the implication of socio-economic development and concomitant lifestyle in the pathophysiology of DM. Hence, lifestyle changes, particularly diet, seem to be an

essential cornerstone of DM management.

In fact, according to the American Diabetes Association (ADA), nutritional therapy is of particular importance in the treatment of DM[5]. Lately, ADA strongly advocates the Mediterranean diet (MD) over other diets because of its beneficial effects on glycaemic control and cardiovascular (CV) risk factors[5]. MD is a characteristic dietary pattern established in the coastal areas of the Mediterranean basin[6]. By definition, MD consists of three to nine servings of vegetables, one to thirteen servings of cereals, half to two servings of fruits, and up to eight servings of olive oil a day[6]. The recommended total fat intake is about 37%, 18% of which are monounsaturated and 9% saturated fats, whereas the recommended fibre intake is 33 g *per* day[6]. Furthermore, moderate consumption of red wine, especially during meals, and rare consumption of red meat are also important factors of this diet[6-8].

Pathophysiology of DM includes a myriad of detrimental molecular processes. Perhaps the critical effector arm of damage in the setting of DM is hyperglycaemia-induced overproduction of reactive oxygen species (ROS). Among multiple other effects, ROS production results in the up-regulated formation of intracellular advanced glycation endproducts (AGEs)[9]. AGEs are typically accumulated slowly in the human body during life, mostly in tissues with slow metabolism[10]. This process is accelerated in some diseases such as DM, renal failure, and various CV diseases[10]. Previous studies have shown that the values of AGEs are elevated in patients with DM and are at least in part the cause of DM-associated CV complications[11,12]. The receptors for advanced glycation endproducts (RAGEs) are found on inflammatory cells (T-lymphocytes and macrophages), endothelial cells and vascular smooth muscle cells. The binding of AGEs to RAGEs stimulates the release of proinflammatory cytokines and growth factors from macrophages, the formation of ROS from the endothelium, the procoagulant action of endothelium and macrophages, as well as interconnection of extracellular matrix proteins[10]. Previous studies have clearly shown that AGE quantity measured from skin tissue is a solid and independent predictor of CV complications, as well as CV morbidity and mortality in DM[13-15]. Unlike skin biopsy, AGE Reader recently emerged as a non-invasive yet reliable instrument for the measurement of AGEs tissue levels[14]. In addition, it has been shown that the AGEs reader has a significant additional value in determining the degree of CV risk in patients with DM[14,15]. In a study by Li *et al*[16], it was demonstrated that patients who had higher values of AGEs also had higher values of glycated hemoglobin (HbA1c), whereas the results of a cross-sectional study by Couppé *et al*[17] show that different long-term exercise regimens can slow down the normal process of AGEs accumulation[16,17].

Although MD and AGEs levels have already been investigated in patients with DM, there is scarce information regarding their mutual interconnections. Hence, the main aim of this study was to examine the association between adherence to the MD and CV risk/AGEs skin levels, in patients with DM type II[18,19]. In addition, we examined the association of anthropometric characteristics, glycaemic control, and physical activity with AGEs levels in the same group of patients. Finally, we examined dietary attitudes and characteristics of the studied population according to DM management. In this study, we hypothesized that patients with DM type II who adhere more to the MD would have lower AGEs skin levels, reflecting lower CV risk.

MATERIALS AND METHODS

Study design and ethical considerations

This study was performed at the Regional Center for Diabetes, Endocrinology and Metabolic Diseases of the University of Split School of Medicine from 1 March 2019 to 1 June 2019. The study was approved by the Ethics Committee of the University of Split School of Medicine. The researchers informed the participants about the procedures and purpose of the study, after which all involved participants signed informed consent.

Subjects

A total of 273 patients (137 men and 136 women) with DM type II participated in this study. DM type II was diagnosed according to the criteria of the ADA[1]. All included participants were treated at the Regional Center for Diabetes, Endocrinology and Metabolic Diseases of the University Hospital of Split, and were interviewed during regular check-ups by a diabetologist. The inclusion criteria was that DM type II is diagnosed for at least a year. On the other hand, newly diagnosed DM type II, DM

type I and other types of diabetes were excluded from the study. For the present study, we initially screened 347 patients. Nineteen patients were excluded because their DM type II diagnosis was established in the past year, 37 patients were excluded because they had DM type I, whereas 10 patients were excluded because they had other types of DM. Finally, among 281 eligible patients, eight refused to participate in the study, yielding an overall acceptance rate of 97%. For the purpose of this study, we performed sample size analysis using MedCalc software. We used an estimated difference in the proportion of the adherence to MD of 0.1, with α error set at 0.05 and study power of 90%. The calculated sample size was 158 participants. To ensure additional power to the study, we collected a substantially larger sample of diabetes patients. Relevant clinical information on each participant was collected by taking anamnesis and checking their medical documentation. Participants also completed a survey on their life habits and the MD service score (MDSS) questionnaire to assess adherence to the MD. Finally, all participants were subjected to measurements of relevant anthropometric features and AGEs value using AGEs reader.

Anthropometric measurements

Participants were subjected to the following measurements of anthropometric features: Body weight and height, neck, waist and hip circumference. A calibrated medical scale with an altimeter (Seca, Birmingham, United Kingdom) was used to measure body weight and height. Body mass index (BMI) was calculated by dividing the value of body weight (kg) by the square of the value of body height (m²). Waist circumference was measured at the upright standing position at the midline level between the bottom of the costal arch in the mid-axillary line and the apex of the iliac ridges. In the same position, the hip circumference was measured at the level of the largest circumference of the gluteal muscles, above the line connecting the large trochanters of the femur. The waist-to-hip ratio is determined by dividing the waist circumference (cm) by the hip circumference (cm). Neck circumference was measured at the midway of the neck, between the mid-cervical spine and mid anterior neck. All circumferences were measured using the same centimetre ribbon with 0.5 cm precision.

Survey

After an extensive literature review, a survey questionnaire that included 27 items divided into three main parts was compiled.

The first part consisted of 7 items that included general patients' information and basic parameters of their disease. The questions included were about: Gender, year of birth, physical activity, smoking status, year of DM diagnosis, last HbA1c levels, and type of DM therapy.

The second part of the questionnaire was MDSS, a useful instrument often used to measure overall diet quality according to the principles of the MD[20]. MDSS is based on the recommended intake of 14 food groups in the Mediterranean food pyramid [18]. While scoring the questionnaire, three points were awarded to those participants who met the appropriate recommended frequency of consumption of the following food groups: Fruits, vegetables, cereals (bread, pasta, rice), and olive oil. Two points were given to those who met the appropriate recommended frequency of dairy products and nuts consumption. Finally, one point was given to those who met the appropriate recommended frequency of consumption of potatoes, legumes, fish, eggs, red meat, white meat, sweets and wine. A score of zero was given if the number of servings of a particular food did not meet the MD recommendations. With this scoring, most points (3 points) are earned by consuming enough food that should be an integral part of every meal in the MD diet. The rest of the points are awarded for foods recommended for intake on a daily (2 points) or weekly (1 point) basis. The overall score can range from 0 to 24 points. A score of over 13.5 points is considered as good adherence to MD principles. The reliability and validity of the Croatian version of the 14-item MDSS questionnaire in assessment of adherence to MD was evaluated by Marendić *et al*[18].

Finally, the third questionnaire part included 6 items that investigated the dietary attitudes of the investigated population. All of the included statements could be answered with "yes" or "no ". Statements were about the source of information about diet, nutritionist support, getting diet information off the internet, the importance of diet in managing personal health problems, usefulness, and attendance of diet-related educational programs.

Measurement of AGEs

AGEs value and associated CV risk were measured using AGE Reader (DiagnOptics

Technologies BV, Groningen, The Netherlands). This is a non-invasive desktop device that uses the characteristic fluorescence of certain AGEs to calculate the level of accumulated AGEs in the skin. This method was confirmed to be firmly consistent with the measurement of the accumulation of AGEs in a skin biopsy sample, taken from the same site where the autofluorescence reading was performed[13].

Participants were briefly introduced to the method of measurement. There was no risk for our participants during this measurement. They were then asked to place their right forearm on the device, which is a standard and practical measuring point on the body for calculating the autofluorescence of certain AGEs from the skin[13]. A series of three consecutive measurements were performed. The results were summed and divided to obtain the mean, which was later used in the analysis. Based on the association of the obtained result with the age of the subjects, a value that classifies participants into one of the four groups (none, limited, increased, definite) depending on their CV risk was calculated. We further stratified these groups into two CV risk groups. The first group included patients with none and limited CV risk, whereas the second included patients with increased and definite CV risk. Finally, this method is observer-independent and has an intrapersonal coefficient of variation of less than 5% [14].

Statistical analysis

Statistical analysis was performed with MedCalc package (version 19.1.2, MedCalc Software, Ostend, Belgium). Categorical variables were presented as whole numbers and percentages, while differences were evaluated with the chi-squared test and Fisher's exact test. Continuous variables were presented as mean and standard deviation or median and interquartile range, according to the normality of data distribution analysis performed with D'Agostino-Pearson test. Accordingly, differences between groups were evaluated with Mann Whitney U-test and t-test for independent samples. Furthermore, the correlation between MDSS score and other selected variables was tested with Spearman's correlation coefficient, while independent factors associated with MDSS scores were evaluated with multiple linear regression analysis. For this purpose, enter algorithm was used, with a report of unstandardized beta coefficients (β), t-values, standard errors (SE), and P-values. The selected model had all assumptions in using multiple regression satisfied. Statistical significance was set at $P < 0.05$.

RESULTS

The study enrolled a total of 273 DM type II patients, with overall AGEs levels of 3.0 (2.6-3.5). Significantly higher values of AGEs were found in men when compared with women [3.1 (2.7-3.7) *vs* 2.9 (2.5-3.3), $P < 0.001$], while HbA1c values were without significant difference between genders [7.3 (6.7-8.8)% *vs* 7.5 (6.7-8.8)%, $P = 0.475$]. According to AGEs levels, patients were divided into none/limited CV risk group ($n = 99$) and increased/definite CV group ($n = 174$). According to these groups, increased CV risk group had significantly higher percentage of male patients (55.2% *vs* 41.4%, $P = 0.029$), and higher levels of HbA1c [7.65 (6.8-8.8)% *vs* 7.2 (6.5-8.5)%, $P = 0.041$]. Other baseline parameters according to the CV risk can be found in Table 1.

According to the MDSS questionnaire, a total of 27 (9.9%) patients fulfilled criteria for adherence to MD, with a median score of 8.0 (6.0-10.0). Patients with none/limited CV risk had significantly higher percentage of those who adhered to MD in comparison to patients with increased/definite CV risk (15.2% *vs* 6.9%, $P = 0.028$). In addition, those participants who followed the MDSS guidelines for nuts (23.2% *vs* 12.6%, $P = 0.023$) and legumes (40.4% *vs* 25.9%, $P = 0.013$) consumption also had significantly higher adherence (Table 2). Furthermore, analysis of MDSS according to DM type II management has shown that significantly higher number of patients with HbA1c < 7 % adhere to MD when compared to patients with HbA1c > 7 % (14.9% *vs* 7.3%, $P = 0.045$). Moreover, those patients follow the guidelines for eggs (33.0% *vs* 46.8%, $P = 0.025$) and wine (15.6% *vs* 29.8%, $P = 0.006$) consumption more frequently (Table 3).

According to gender, women followed the guidelines for red meat consumption more frequently than men (34.3% *vs* 58.8%, $P < 0.001$), while men had better follow of guidelines for cereals (70.8% *vs* 55.9%, $P = 0.011$) and wine (33.6% *vs* 7.4%, $P < 0.001$) consumption. Adherence to all food groups in MDSS according to gender can be found in Table 4.

Parameter	None/limited CV risk (*n* = 99)	Increased/definite CV risk (*n* = 174)	Total (*n* = 273)	*P* value[1]
Table 1 Baseline parameters analysis according to cardiovascular risk groups derived from advanced glycation endproducts result				
Male gender, *n* (%)	41 (41.4)	96 (55.2)	137 (50.2)	0.029
Age (yr)	67.6 ± 12.4	69.1 ± 11.8	68.5 ± 12.1	0.313
Disease duration (yr)	9.0 (3.0-18.0)	14.0 (6.0-21.0)	12.0 (5.0-20.0)	0.005
BMI (kg/m^2)	29.2 ± 4.96	29.1 ± 4.85	29.2 ± 4.88	0.900
Neck circumference (cm)	38.7 ± 4.01	39.1 ± 4.17	38.9 ± 4.11	0.409
Waist circumference (cm)	98.1 ± 12.7	97.8 ± 12.7	97.9 ± 12.6	0.870
Hip circumference (cm)	106.3 ± 9.5	105.5 ±10.5	105.8 ± 10.1	0.531
WHR	0.92 ± 0.07	0.93 ± 0.08	0.92 ± 0.08	0.575
Smokers, *n* (%)	12 (12.1)	41 (23.6)	53 (19.4)	0.022
Physical activity, *n* (%)				
Not physically active	15 (15.2)	56 (32.2)	71 (26.0)	0.008
1-4 ×/mo	24 (24.2)	36 (20.7)	60 (22.0)	
> 4 ×/mo	60 (60.6)	82 (47.1)	142 (52.0)	
Therapy, *n* (%)				
OHA	56 (56.6)	90 (51.7)	146 (53.5)	0.619
Insulin	14 (14.1)	26 (14.9)	40 (14.7)	
OHA + insulin	23 (23.2)	49 (28.2)	72 (26.4)	
GLP-1 based therapy	2 (2.0)	6 (3.4)	8 (2.9)	
No medications	4 (4.0)	3 (1.7)	7 (2.6)	
HbA1c (%)	7.2 (6.5-8.5)	7.65 (6.8-8.8)	7.4 (6.7-8.8)	0.041

[1]Chi-square test, Mann-Whitney test/t-test for independent samples. Data are presented as whole numbers (%), median (IQR) or mean ± SD. Bolded parameters signify statistically significant difference. AGE: Advanced glycation endproducts; OHA: Oral hypoglycemic agents; GLP: Glucagon-like peptide; HbA1c: Glycated haemoglobin.

Further analyses have shown that MDSS score has a significant positive correlation with disease duration (*r* = 0.179, *P* = 0.003) and negative correlation with BMI values (*r* = -0.159, *P* = 0.008) (Table 5). Moreover, in the multiple linear regression model, BMI (β ± SE, -0.09 ± 0.04, *P* = 0.037) and disease duration (β ± SE, 0.07 ± 0.02, *P* < 0.001) remained significant independent correlates of MDSS score (Table 6).

Finally, attitudes regarding diet and diet behaviours were analysed. Most of the patients that get diet information from a physician (96.3%), think that a better and more controlled diet could reduce their health problems (88.6%) and would visit educational programs if they existed (81.7%). Furthermore, advice from a nutritionist was received in 16.8%of cases, while 17.9% of patients have informed themselves on the Internet regarding diet in diabetes. According to DM management, patients with worse DM control think that educational programs on nutrition would be helpful, in significantly more cases in comparison to patients with better control of DM (98.9% *vs* 92.6%, *P* = 0.009). Dietary attitudes of the studied population according to DM management can be seen in Table 7.

DISCUSSION

In the present cross-sectional study, we demonstrated that DM type II patients with no or limited CV risk adhere more to the MD than patients with either increased or definite CV risk. In line with this, significantly more patients with none or limited CV risk followed the recommended use of nuts and legumes. Furthermore, we showed that the subgroup of DM type II patients with lower HbA1c levels (< 7%) adheres better to the MD than the subgroup of patients with higher HbA1c levels (> 7%), markedly in terms of following the recommended use of wine and eggs. In addition,

Table 2 Adherence to individual food groups and total Mediterranean diet guidelines according to cardiovascular risk groups derived from advanced glycation endproducts levels

Parameter, *n* (%)	None/limited CV risk (*n* = 99)	Increased/definite CV risk (*n* = 174)	Total (*n* = 273)	*P* value[1]
Cereals	60 (60.6)	113 (64.9)	173 (63.4)	0.475
Potato	87 (87.9)	153 (87.9)	240 (87.9)	0.989
Olive oil	28 (28.3)	45 (25.9)	73 (26.7)	0.664
Nuts	23 (23.2)	22 (12.6)	45 (16.5)	0.023
Fresh fruit	36 (36.4)	57 (32.8)	93 (34.1)	0.546
Vegetables	10 (10.1)	13 (7.5)	23 (8.4)	0.453
Milk and dairy products	14 (14.1)	44 (25.3)	58 (21.2)	0.031
Legumes	40 (40.4)	45 (25.9)	85 (31.1)	0.013
Eggs	40 (40.4)	63 (36.2)	103 (37.7)	0.492
Fish	32 (32.3)	50 (28.7)	82 (30.0)	0.535
White meat	34 (34.3)	53 (30.5)	87 (31.9)	0.509
Red meat	39 (39.4)	88 (50.6)	127 (46.5)	0.075
Sweets	77 (77.8)	137 (78.7)	214 (78.4)	0.854
Wine	19 (19.2)	37 (21.3)	56 (20.5)	0.684
Total MDSS points	7.0 (6.0-10.75)	8.0 (6.0-10.0)	8.0 (6.0-10.0)	0.959

[1]Chi-square test or Mann-Whitney test. Data are presented as whole numbers (%) or median (IQR). Bolded parameters signify statistically significant difference. MDSS: Mediterranean diet serving score.

these subgroups mainly did not differ in dietary attitudes. Interestingly, MDSS correlated positively with disease duration and negatively with BMI, which was further confirmed in a multiple linear regression model. Finally, certain gender differences in diet adherence were also observed, as women followed guidelines for red meat consumption more frequently than men, whereas it was vice versa with respect to guidelines for cereals and wine.

Our results regarding the association between MD adherence and CV risk are in concordance with the available studies. Namely, it has so far been well established that adherence to MD improves CV outcomes[21-24]. Among an array of the conducted studies, the PREDIMED (*PREvención con DIeta MEDiterránea*) trial should be specially addressed, as insights from it provided a large body of evidence on the association between MD and diverse health outcomes[25]. The PREDIMED is a multicentre, randomized, primary prevention trial that included 7447 participants aiming to assess the long-term effects of MD on the occurrence of CV events. The trial clearly showed a 30% relative risk reduction in CV event incidence, while additionally calculating that, in a 5-year follow-up, in a hypothetic cohort of 1000 people following the MD, 13 CV events can be prevented. Notably, accounting for the advanced age of the study participants, it has been concluded that it is never too late to improve dietary regiments in terms of CV health. The main advantage of the PREDIMED trial was a continuous assessment of MD adherence, unlike most of the other follow-up studies in which dietary habits were only measured at the commencement of the study. However, it is important to point out that no effect on all-cause mortality was apparent in this trial, unlike other studies conducted on this topic[26-28]. Pathophysiological mechanisms by which MD exhibits the above-noted protective effects on the CV system are diverse[29]. Although not completely elucidated, the richness of MD nutrients in anti-oxidant and anti-inflammatory molecules is likely to be relevant[30, 31]. Nutrients can either have intrinsic anti-oxidant capacity or modulate gene and protein expression. Available studies suggest that MD exerts protective effects on the expression of several proatherogenic genes, resulting in modulation of vascular inflammation, thrombosis, and foam-cell formation[32-34]. In this sense, legumes, for which we demonstrated that patients with lower CV risk have a higher adherence, contain a lot of phytochemicals endowed with useful biological activities[35,36]. Phytochemicals were shown to have prominent antioxidant activity, improve endothelial function by increasing nitric oxide bioavailability and prevent athero-

Table 3 Adherence to individual food groups and total Mediterranean diet guidelines according to diabetes management

Parameter, *n* (%)	HbA1c > 7 % (*n* = 179)	HbA1c < 7 % (*n* = 94)	Total (*n* = 273)	*P* value[1]
Cereals	113 (63.1)	60 (63.8)	173 (63.4)	0.909
Potato	159 (88.8)	81 (86.2)	240 (87.9)	0.523
Olive oil	48 (26.8)	25 (26.6)	73 (26.7)	0.969
Nuts	27 (15.1)	18 (19.1)	45 (16.5)	0.390
Fresh fruit	57 (31.8)	36 (38.3)	93 (34.1)	0.286
Vegetables	15 (8.4)	8 (8.5)	23 (8.4)	0.971
Milk and dairy products	38 (21.2)	20 (21.3)	58 (21.2)	0.993
Legumes	50 (27.9)	35 (37.2)	85 (31.1)	0.115
Eggs	59 (33.0)	44 (46.8)	103 (37.7)	0.025
Fish	51 (28.5)	31 (33.0)	82 (30.0)	0.443
White meat	60 (33.5)	27 (28.7)	87 (31.9)	0.419
Red meat	86 (48.0)	41 (43.6)	127 (46.5)	0.487
Sweets	144 (80.4)	70 (74.5)	214 (78.4)	0.255
Wine	28 (15.6)	28 (29.8)	56 (20.5)	0.006
Total MDSS points	8.0 (6.0-10.0)	8.0 (6.0-11.0)	8.0 (6.0-11.0)	0.261

[1]Chi-square test or Mann-Whitney test. Data are presented as whole numbers (%) or median (IQR). Bolded parameters signify statistically significant difference. MDSS: Mediterranean diet serving score.

sclerosis progression by inhibition of low-density lipoprotein oxidation[37,38]. Accordingly, nutrients in nuts, for which we demonstrated better adherence among low-risk CV subgroup as well, are also associated with multiple molecular pathways which grant their beneficial CV effects. Namely, randomized trials have proven that nuts consumption is associated with attenuation of inflammation and oxidative stress burden, improvement in endothelial function and lipid status, as well as in insulin resistance[39,40].

The rationale for using AGE Reader in CV risk stratification is substantiated by multiple evidence[41]. AGEs serum or plasma levels do not reflect levels of AGEs in tissues, owing to the high protein turnover rate in the circulation[42]. Conversely, AGE Reader reliably reflects AGEs tissue levels obtained using skin biopsy by utilizing fluorescent properties that several AGEs possess[43]. Furthermore, multiple studies indicate that accelerated accumulation of AGEs was proportionally associated with higher CV risk, thus justifying the use of AGE in this setting[13,14,15,44].

Effects of MD adherence on glycaemic control in DM type II were thoroughly reviewed by Esposito *et al*[45]. Multiple authors provided evidence which points to an inverse association between MD and indices of glucose homeostasis in the general population, the elderly, and high-risk patients[46-48]. Among five conducted RCTs that evaluated the effects of MD on glycaemic control in DM type II[49-53], only one trial showed no difference in HbA1c levels between the control group[49]. Namely, Shai *et al*[49] compared three weight-loss diets in 322 moderately obese patients, 46 of which suffered from DM type II, and demonstrated no difference in HbA1c decrease between the groups assigned to the MD as compared with the low-fat diet, however, a significant decrease in fasting glucose concentration was found in the MD cohort. Our results seem to be in line with findings from the aforementioned studies. In addition, more optimal adherence for wine and eggs in the subgroup with lower HbA1c was observed in the present study. This observation could be explained by the fact that wine and eggs were both shown to exert favourable effects on the metabolic profile in patients with DM[54,55].

Although our findings that adherence to MD positively correlates with disease duration seem reasonable, conflicts with some of the available data. Austin *et al*[56] demonstrated that DM type I duration is indicative of poorer dietary self-care in adolescents[56]. Authors attributed this observation to contextual and motivational factors as posited by Self-Determination Theory. However, owing to the significant age difference and concomitant differences in psychological features between ours and

Table 4 Adherence to individual food groups and total Mediterranean diet guidelines according to gender

Parameter, n (%)	Men (n = 137)	Women (n = 136)	Total (n = 273)	P value[1]
Cereals	97 (70.8)	76 (55.9)	173 (63.4)	0.011
Potato	120 (87.6)	120 (88.2)	240 (87.9)	0.871
Olive oil	37 (27.0)	36 (26.5)	73 (26.7)	0.920
Nuts	18 (13.1)	27 (19.9)	45 (16.5)	0.135
Fresh fruit	42 (30.7)	51 (37.5)	93 (34.1)	0.234
Vegetables	9 (6.6)	14 (10.3)	23 (8.4)	0.269
Milk and dairy products	33 (24.1)	25 (18.4)	58 (21.2)	0.250
Legumes	38 (27.7)	47 (34.6)	85 (31.1)	0.224
Eggs	59 (43.1)	44 (32.4)	103 (37.7)	0.068
Fish	43 (31.4)	39 (28.7)	82 (30.0)	0.626
White meat	43 (31.4)	44 (32.4)	87 (31.9)	0.864
Red meat	47 (34.3)	80 (58.8)	127 (46.5)	< 0.001
Sweets	103 (75.2)	111 (81.6)	214 (78.4)	0.197
Wine	46 (33.6)	10 (7.4)	56 (20.5)	< 0.001
Total MDSS points	8.0 (6.0-10.0)	8.0 (6.0-11.0)	8.0 (6.0-10.0)	0.485
Adherence to Mediterranean Diet	12 (8.8)	15 (11.0)	27 (9.9)	0.530

[1]Chi-square test or Mann-Whitney test. Data are presented as whole numbers (%) or median (IQR). Bolded parameters signify statistically significant difference. MDSS: Mediterranean diet serving score.

Table 5 Correlation of Mediterranean diet serving score with selected parameters in study population (n = 273)

Parameter	MDSS score, r (P value[1])
Age (yr)	0.054 (0.372)
Disease duration (yr)	0.179 (0.003)
BMI (kg/m^2)	-0.159 (0.008)
Neck circumference (cm)	-0.001 (0.991)
Waist circumference (cm)	-0.010 (0.872)
Hip circumference (cm)	-0.017 (0.779)
WHR	-0.022 (0.723)
HbA1c (%)	-0.074 (0.220)
AGEs levels	-0.012 (0.839)

[1]Spearman's correlation coefficient. AGE: Advanced glycation endproducts; MDSS: Mediterranean diet serving score; BMI: Body mass index; WHR: Waist-to-hip ratio; HbA1c: Glycated hemoglobin.

Austin *et al*[56]'s study participants, their findings could hardly be extrapolated to the current study. On the other hand, multiple studies conducted on populations similar to the population present in this study also demonstrated opposite results. Most authors agree that strict dietary habits fade over time, as DM patients are under the constant threat of severe diabetic complications despite being adherent to self-care behaviours, which results in burnout[57,58]. As overall MD adherence was low in the current, but in other studies as well, a search for novel educational techniques is warranted[59-61]. This is also recognized by study participants (97%), markedly in those with higher HbA1c levels. These results may be explained by multiple factors. In both our and other studies, the first and probably the main culprit for low MD adherence are overall high prices for dietary products of which MD is comprised.

Table 6 Multiple linear regression model of independent predictors for Mediterranean diet service score

Variable	B[1]	SE[2]	*t* value	*P* value
Gender	-0.39	0.43	-0.92	0.354
Age (yr)	-0.002	0.02	-0.13	0.895
Disease duration (yr)	0.07	0.02	3.42	< 0.001
Body mass index (kg/m^2)	-0.09	0.04	-2.11	0.037
HbA1c (%)	-0.07	0.13	-0.59	0.554
AGEs levels	-0.31	0.31	-1.05	0.311

[1]Unstandardized coefficient β.

[2]Standard error.

AGE: Advanced glycation endproducts; HbA1c: Glycated hemoglobin.

Table 7 Dietary attitudes of studied population according to diabetes management

Questions	HbA1c > 7% (*n* = 179)	HbA1c < 7% (*n* = 94)	Total (*n* = 273)	*P* value[1]
Did you get diet information according to your illness from your physician (yes)	172 (96.1)	91 (96.8)	263 (96.3)	0.764
Have you visited a nutritionist to advise you on nutrition (yes)	26 (14.5)	20 (21.4)	46 (16.8)	0.157
Have you informed yourself on the Internet about the diet related to your illness (yes)	29 (16.2)	20 (21.4)	49 (17.9)	0.300
Do you think that a better and more controlled diet could reduce your health problems (yes)	156 (87.2)	86 (91.5)	242 (88.6)	0.331
Do you consider educational programs on nutrition to be useful for patients (yes)	177 (98.9)	87 (92.6)	264 (96.7)	0.009
If educational programs on nutrition existed in your community, would you visit them (yes)	145 (81.0)	78 (83.0)	223 (81.7)	0.689

Data are presented as whole numbers (%).

[1]Chi-square test.

HbA1c: Glycated hemoglobin.

Furthermore, the westernization of society has also impacted the dietary preferences of the population, shifting to high-sugar, low-fibre processed food. Finally, the knowledge concerning healthy dietary habits in our population is generally low, even among general practitioners[62,63]. Given that only 16% of our participants underwent nutritionist evaluation despite that average disease duration was 12 years, this could be a promising niche for diet improvement in patients with DM, as demonstrated by multiple authors[64,65]. Conversely, a negative correlation was found between MD adherence and BMI. These results are expected, as MD adherence was previously shown to participate in weight reduction[66,67]. In addition, patients who are more motivated for dietary self-care are more prone to weight loss[67].

The observed decrease in consumption of red meat in female participants and increased consumption of wine and cereals among men is in line with the available data[66]. Multiple authors suggest that gender differences in adherence to certain food groups could be explained by psychological dissimilarities between men and women, more frequent veganism in woman populations, and reduced accessibility of meat to women[68,69]. Results from the present study imply that patients with higher AGEs levels and concomitantly higher CV risk are less eager to remain physically active. The obtained results are in concordant with the available data, as most studies demonstrated that physical inactivity is associated with high levels of AGEs and other predictors of CV risk[70,71]. Interestingly, in a study by Rodrigues *et al*[72], the authors demonstrated that low AGEs levels in patients with human immunodeficiency virus could be restored to normal values by exercising in just a few months[72].

Our study bears several limitations. Firstly, the cross-sectional design of the study which prevents us from making any causal inferences. Secondly, in the questionnaire, we have relied on the participant's recall memory, therefore, the answers are

susceptible to subjectivity and recall bias. Finally, although considered reliable, we must bear in mind that AGE Reader has many different endogenous and exogenous components which may influence measurement of the AGEs.

CONCLUSION

In summary, this cross-sectional study brought further evidence concerning the association between AGE tissue levels and adherence to MD using a validated MDSS questionnaire. In line with this, we demonstrated that adherence to MD is very low in patients with DM type II, especially in those with poorly controlled glycaemia. Nevertheless, survey results indicate that patients seem to realize the importance of diet in DM management. Furthermore, it has been observed that dietary preferences are influenced by gender, women followed guidelines for red meat consumption more frequently than men, whereas it was vice versa in guidelines for cereals and wine. Finally, we showed that DM duration independently predicted better adherence to MD, whereas BMI predicted poorer MD adherence.

ARTICLE HIGHLIGHTS

Research background

In recent years, major diabetic organizations started to strongly advocate the use of the Mediterranean diet (MD) over other diets in patients with diabetes mellitus (DM) because of its beneficial effects on glycaemic control and cardiovascular (CV) risk factors. Evidence suggests that CV risk may be assessed using tissue levels of advanced glycation endproducts (AGEs) in patients with DM.

Research motivation

As DM prevalence is constantly rising in well-developed countries, there is an urgent need to mitigate the poor outcomes of this disease. Regarding the importance of diet in this setting, we endeavoured to bring further evidence with respect to the benefits of the use of the MD in patients with DM.

Research objectives

The main objective of this study was to examine the association between adherence to the MD, assessed by MD serving score (MDSS) and CV risk, assessed by AGEs skin levels, in patients with DM type II. Additionally, we examined the association between anthropometric characteristics, glycaemic control, and physical activity with AGEs levels among patients with DM type 2

Research methods

In this study, we employed the Croatian version of the 14-item MDSS questionnaire to assess adherence to the MD. On the other hand, in order to compare adherence to CV risk, we used skin autofluorescence-based AGE Reader that measures AGEs skin levels.

Research results

The present study demonstrated that patients with diabetes who have none or limited CV risk adhere more to the MD than patients who have either increased or definite CV risk. In addition, we showed that the subgroup of patients with diabetes with better glycaemic control adheres better to the MD than the subgroup of patients with worse glycaemic control. Altogether, these results are generally in line with the available data. It remains to be answered why adherence to MD is so low, despite being undoubtedly beneficial.

Research conclusions

By bringing additional data about the association of the MD with CV outcomes, this study addresses the need to implement novel strategies that will lead to better MD adherence in patients with diabetes.

Research perspectives

In future studies, the highlight should be placed further delineation off mechanisms by

which MD exerts its favourable effects to establish the optimal dietary pattern. Furthermore, psychological studies could be important in this setting, as the main problem of MD is low adherence. Namely, psychological studies may give a deeper insight into non-adherence, thus facilitating the resolution of this issue.

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Observational Study

Comprehensive genetic screening reveals wide spectrum of genetic variants in monogenic forms of diabetes among Pakistani population

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Abstract

BACKGROUND
Monogenic forms of diabetes (MFD) are single gene disorders. Their diagnosis is challenging, and symptoms overlap with type 1 and type 2 diabetes.

AIM
To identify the genetic variants responsible for MFD in the Pakistani population

and their frequencies.

METHODS
A total of 184 patients suspected of having MFD were enrolled. The inclusion criterion was diabetes with onset below 25 years of age. Brief demographic and clinical information were taken from the participants. The maturity-onset diabetes of the young (MODY) probability score was calculated, and glutamate decarboxylase ELISA was performed. Antibody negative patients and features resembling MODY were selected (*n* = 28) for exome sequencing to identify the pathogenic variants.

RESULTS
A total of eight missense novel or very low-frequency variants were identified in 7 patients. Three variants were found in genes for MODY, *i.e. HNF1A* (c.169C>A, p.Leu57Met), *KLF11* (c.401G>C, p.Gly134Ala), and *HNF1B* (c.1058C>T, p.Ser353Leu). Five variants were found in genes other than the 14 known MODY genes, *i.e. RFX6* (c.919G>A, p.Glu307Lys), *WFS1* (c.478G>A, p.Glu160Lys) and *WFS1* (c.517G>A, p.Glu173Lys), *RFX6* (c.1212T>A, p.His404Gln) and *ZBTB20* (c.1049G>A, p.Arg350His).

CONCLUSION
The study showed wide spectrum of genetic variants potentially causing MFD in the Pakistani population. The MODY genes prevalent in European population (*GCK, HNF1A, and HNF4a*) were not found to be common in our population. Identification of novel variants will further help to understand the role of different genes causing the pathogenicity in MODY patient and their proper management and diagnosis.

Key Words: MODY; Diabetes; Genetics; Monogenic diabetes; Monogenic forms of diabetes

Core Tip: There was a lack of data on monogenic forms of diabetes (MFD) from Pakistan, therefore this study was designed to determine the genetic variants responsible for MFD in the country. The study identified wide spectrum of genetic variants potentially causing MFD. The identification of novel variants paved the way for better understanding of genetic landscape and risk factors of MFD in the country.

INTRODUCTION

Monogenic forms of diabetes (MFD) result from changes in single gene. Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes. It is inherited in an autosomal dominant pattern[1] and is often misdiagnosed as type 1 or type 2 diabetes[2]. It is estimated that MODY accounts for 1%-2% of all the diabetic cases[3]. There are fourteen sub-types of MODY listed in OMIM(On-Line Mendelian Inheritance in Man) (#606391). Most common of them are *GCK, HNF1A, and HNF4A*. The other listed genes are *PDX1, HNF1B, NEUROD1, KLF11, CEL, PAX4, INS, BLK, ABCC8, KCNJ11,* and *APPL1*[1]. Mutations in a number of additional genes are also known to cause diabetes.

The subtypes of MODY differ with respect to hyperglycemia, age of disease onset, treatment pattern, and complications reported[4].Therefore timely diagnosis is of vital importance in MFD. Previously, most common genes, *i.e. GCK, HNF1A,* and *HNF4A* were generally sequenced for suspected cases, but n ow with the advent of latest technologies, targeted panel sequencing or exome sequencing are standard[5,6].

Pakistan has a huge burden of diabetes. The recent surveys showed that one out of every four people in the general population is suffering from diabetes in the country[7, 8]. Being a resource poor country, advanced diagnostic facilities are not available to the public. The literature from Pakistan is scarce on MFD[9,10]. Therefore, this study was designed to enroll suspected MFD patients and assess the causal variants involved. To our knowledge, this was the first comprehensive study to be conducted in Pakistan on MFD.

MATERIALS AND METHODS

A total of 184 patients with diabetes onset before 25 years of age were considered for participation in the study. The participants were chosen from the sources indicated below:

National Diabetes Survey (n = 80)

The record of patients who had an onset of disease before 25 years of age was obtained from National Diabetes Survey data. This was a population-based survey carried out all over the four provinces of Pakistan. The detailed methodology is described elsewhere[7]. According to World Health Organization, the patients were diagnosed as diabetic if having fasting glucose level > 126 mg/dL (7.0 mmol/L) or HbA1c > 6.5% (48 mmol/mol). Clinical information in 34 cases suggested MFD [young onset, low body mass index (BMI), mild hyperglycemia]. The glutamate decarboxylase 65 (GAD-65) ELISA testing was negative in 5 cases, which were selected for exome sequencing.

Prospective enrollment from Lahore (n = 15)

A total of 15 patients were enrolled from The Diabetes Clinic at PHRC Research Centre, Fatima Jinnah Medical University, Lahore. The inclusion criteria were onset of diabetes before 25 years of age with preferential first-degree family history of diabetes. The demographic information along with history of disease (onset, complications, treatment details and family history) was collected on proforma. On the basis of BMI and preserved fasting C-peptide, GAD-65 ELISA was performed on three samples where serum was available. The two that were found negative for GAD-65 and, along with another three patients having clinical features like low BMI but for whom serum was not available, were finally selected for exome sequencing. All the selected subjects had normal c-peptide values (range: 0.8-3.8 ng/mL)

Prospective enrollment from Karachi (n = 89)

The patients coming to the diabetes clinic for type-1 and type 2 diabetes treatments were enrolled if onset of the disease was below 25 years of age, with family history of diabetes as preference. Information on demography, treatment, and diagnosis was taken on pre-designed proforma. Height and weight were recorded for BMI. The GAD-65 ELISA was performed on 59 patients. Patients that were GAD-65 negative and additional patients with low BMI but no available serum for testing were selected for exome sequencing (*n* = 18) (Figure 1).

A total of 28 patients were selected for exome sequencing. Their median age at diagnosis was 18 years and median BMI was 22. Among them, 17 were taking treatment (14 insulin and 3 were taking OHA agent) and 5 were not taking any treatment.

The DNA was extracted using a QIAamp DNA mini kit (QIAGEN). The GAD-65 autoantibody test was done by using a KRONUS Elisa kit. C-peptide test was commercially tested in a diagnostic laboratory. Exome sequencing was performed by Genome Quebec, Canada. Exome sequencing was carried out with 50 Mb Agilent Sure select array and sequencing on Illumina Hi-seq at 50 × depth.

Written informed consent was taken from participants/parents/guardians prior to enrollment. The Ethical Clearance was taken from Institutional Bioethics Committee of Pakistan Health Research Council and Ethical Review Board of Pakistan Institute of Medical Sciences, Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad.
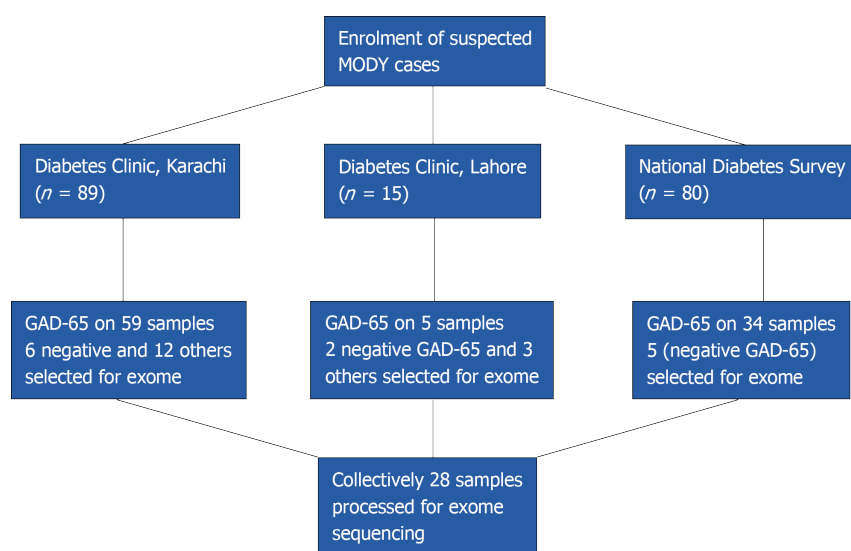
Figure 1 Flow chart for enrollment of patients. GAD-65: Glutamate decarboxylase.

Data analysis

For bioinformatics analysis, the FastQ files were processed using the best practices recommendations of the genome analysis tool kit (GATK). The reads were mapped to human reference genome GRCh37 using Burrow-Wheeler alignment (BWA-MEM). The Picard tool was used to mark and remove duplicate alignments and then indel realignment and base quality score recalibration was done. The gVCFs were generated with GATK HaplotypeCaller and joint variant calling was done. The variants with low map reads (DP < 20) and low genotype quality (GQ < 20) were discarded. Annovar was used to annotate the variants. UTR, synonymous, and intronic (unless splicing) variants were discarded by standard procedure to focus on protein altering ones. These variants were filtered for frequency in three public databases (Exome Aggregation Consortium, 1000 genomes, and gnomAD version v2.1.1). For dominant genes, maximal allele frequency cutoff was 0.0001 in any population while it was 0.005 for recessive genes (ACMG PM2 criterion). The missense variants were selected only if predicted disease-causing by the majority of 10 algorithms used (see legend to Table 1) which satisfies PP3 by ACMG/AMP criteria. The computational evidence supports deleterious effects on the gene[11]. Analysis of the exome focused on the genes listed in the University of Chicago monogenic diabetes panel (https://dnatesting.uchicago. edu/tests/monogenic-diabetes-panel). Variant coordinates were searched for functional domains in uniport.org (results shown in Table 1).

RESULTS

A total of 80 cases selected from the National Diabetes Survey with the criteria of having diabetes and onset of disease before 25 years of age. The median age was 24 years. Most of them (62%) were females and average BMI was 28 kg/m^2 (Table 2). Of them, based on clinical criteria and negativity for antibody, five were selected for exome sequencing. The results revealed three novel missense variants identified in three patients. Two of the variants belong to the OMIM-listed MODY genes, *i.e.* *HNF1A* (c. 169C>A, pLeu57Met) and *KLF11* (c. 401G>C, p.Gly134Ala) and one in *RFX6* (c. 919G>A, p.Glu307Lys), a gene recently described as mutated in dominant, MODY-like diabetes.

In the prospective enrollment from Lahore, 15 patients were enrolled. The mean age for onset of disease in patients was 23 years and mean BMI was 21.5 kg/m^2. The fasting blood glucose on average was 239.26 mg/dL. The HbA1c ranged from 6% to 11% (Supplementary Table 1). The exome analysis revealed a variant in *HNF1B* (c. 1058C>T, p.Ser353Leu) for one patient.

In the prospective enrollment from Karachi, 89 diabetic patients were enrolled. The mean age of onset of disease was 16.5 years and the current age was 26.2 years. Mean BMI was 24.3 kg/m^2. Most of them (95%) had family history of diabetes. Among them, 93% were taking treatment for diabetes and 68% were taking insulin. About 25% of the

Table 1 Rare variants in cases of monogenic forms of diabetes

Case ID	Chromosome position	Gene symbol	cDNA change	Protein change	Maxfreq.	In silico prediction	Protein region byUniport (uniport.org)
612	12; 1416740	*HNF1A*	c. 169C>A	p.Leu57Met	0	T, NA, D, D, N, L, D, D, D, D	DNA-interacting
705	2; 10187916	*KLF11*	c. 401G>C	p.Gly134Ala	0	T, N, N, T, N, N, T, T, T, N	NA
830	6;117237424	*RFX6*	c. 919G>A	p.Glu307Lys	0.0001	T, D, D, T, N, N, T, T, D, D	NA
P-9	17; 6070581	*HNF1B*	c. 1136C>T	p.Ser379Leu	0.00003	NA, D, D, NA, NA, L, D, D, D, D	NA
P-17	4; 6292941	*WFS1*	c. 478G>A	p.Glu160Lys	0	T, N, N, D, N, M, D, D, D, D	NA
	4; 6292980		c. 517G>A	p.Glu173Lys	0	D, D, D, D, D, M, T, T, D, D	
P-68	6; 117241502	*RFX6*	c. 1212T>A	p.His404Gln	0.0001	T, D, D, T, N, L, T, T, T, D	NA
P-87	3; 114069876	*ZBTB20*	c. 1049G>A	p.Arg350His	0	D, D, D, T, N, N, T, T, T, D	NA

Fathmm-MKL_coding are listed in sequence. D: Deleterious; T: Tolerated for SIFT, LRT, MetaSVM, MetaLR_pred, M-Cap prediction, FATHMM; N: Neutral for LRT and Provean. For mutation assessor, H: High; M: Medium; L: Low; N: Neutral; NA: Not available. SIFT: Sorting intolerant from tolerant; LRT: Likelihood ration test; Mutation taster, FATHMM: Functional Analysis through Hidden Markov Models; PROVEAN: Protein Variation Effect Analyzer; Mutation assessor, MetaSVM: Support vector machine; MetaLR: Logistic regression; M-CAP: Mendelian Clinically Applicable Pathogenicity.

Table 2 Demographic features of patients having causal variants

ID	Age at diagnosis	Sex[1]	HbA1c (%)	Affectedparent[2]
612	25	F	9.2	M and F
705	Not listed but current age 23	M	5.4	NA
830	Not listed but current age 21	F	7.2	NA
P-9	20	M	6.20	-
P-17	15	F	NA	M
P68	13	M	6.8	M
P87	25	M	6.10	M

[1]M: Male; F: Female.
[2]M: Mother; F: Father.

patients had a MODY probability score more than 75%. The serum was available for 59 patients. The GAD-65 autoantibody test was conducted on all patients and 15 of them were negative. The patients whose serum was not available were shortlisted on the basis of low BMI, family history, and HbA1c levels.

Among them, 18 were selected for exome sequencing, which revealed potentially causal variants in three patients (Table 1). One had a variant in *RFX6* (c. 1212T>A, p.His404Gln) and a second one was a compound heterozygote (Supplementary Figure 1) for *WFS1* (c. 478G>A, p.Glu160Lys and c. 517G>A, p.Glu173Lys). Recessive *WFS1* mutations cause Wolfram syndrome, but non-syndromic diabetes alone is also seen[12]. A third patient had a variant in *ZBTB20* (c. 1049G>A, p.Arg350His).

The mutation in this gene is responsible for causing primrose syndrome[13]. The patient did not have the other manifestations of this syndrome.

All variants reported here were missense, all satisfied the PP3 and PM2 criteria but, being novel and not having previously been reported, all classified as VUS. Nevertheless, our extremely low cut-off for allele frequency of 0.0001 (more than two orders of magnitude lower than the ACMG/AMP cut-off for a VUS), minimizes the

probability of spurious variants. The MODY probability score was calculated for all the participants. More than half of the patients in National Diabetes Survey, all patients from Lahore and one fourth of patients from Karachi had probability scores more than 75%. The probability score was calculated by MODY probability calculator developed for Caucasian population.

DISCUSSION

Exome sequencing of 28 suspected patients for MFD identified missense novel variants in 7 patients (with the caveat that *KLF11, BLK,* and *PAX4* are not universally accepted as genes whose mutation causes diabetes). Previous studies from Pakistan have discussed the importance of diagnosing MFD in Pakistan[9,10]. However, to the best of our knowledge, this is the first comprehensive study from the country to enroll suspected MFD patients for exome sequencing.

We enrolled diabetic patients with early onset of disease below 25 years of age with clinical features suggestive of MFD and negative (or unknown) for GAD65 auto antibodies. The probability score was calculated by using the MODY probability calculator developed for the Caucasian population[14]. However there is a need to validate this with South Asian populations[15].

In determining the type of MFD, ethnic differences play an important role. There is wide variation of prevalence of different MFD types in different areas. The *HNF1A* MODY type was reported to be more prevalent in northern Europe while *GCK* MODY types in southern Europe[16]. There were similar findings from United States regarding the three major prevalent types of MODY in their population[17]. The study on Russian children with non-type 1 diabetes showed that the most prevalent MODY type was *GCK,* with only 18% of variants in other than known MODY genes. The studies from China reported that *HNF4A* MODY types were relatively less common as compared to Europeans[18,19]. A study from Korea also has a similar finding that common MODY types prevalent in Europe were not common there, but instead they found variants in three new genes, including the *WFS1* gene[20]. The MODY landscape in India is also complex, reporting MODY types other than common genes known in European population[21,22]. A study from Oman reported that variants were not found in three common MODY genes[23]. Similar findings were also reported from Tunisia that common MODY types were not found there and concluded that other genes might be responsible for young onset diabetes in their population[24, 25]. These discrepant results may be only partially explained by different methodologies and different selection criteria for testing.

We found in our study in a total 28 screened patients that 3 of the patients tested had variants in OMIM-listed MODY genes while 4 had variants were in other genes also known to be mutated in MFD. Similar findings were also reported from Norway[26], France[27] and Sweden[28]. Two novel missense variants were found in *RFX6* (c.G919A, p.E307K). In addition to Europeans[29], variants from this gene was also reported in studies from India[22] and Japan[30]. One patient was compound heterozygous at *WFS1*, the gene mutated in Wolfram syndrome and also responsible for non-syndromic diabetes. *WFS1* variants have been reported from India[22], China[12,31], Korea[20,32,33], Russia[6], and European ancestry[34]. Finally, one variant was identified in *ZBTB20*, a transcription factor that regulates the function of beta cells and glucose homeostasis[35-37]. In humans, dominant *ZBTB20* mutations cause Primrose syndrome.

The three variants in OMIM-listed MODY genes were found in *HNF1A*, *KLF11*, and *HNF1B*. The *HNF1A* (MODY 3) is most common type prevalent in some European and Asian countries[38] and variants in this gene have been reported from various countries all around the world[5,39,40]. The patients with *HNF1A* variants respond well to sulfonylurea therapy[41]. The other variant was found in the *KLF11* (MODY 7) [42]; variants from this gene have been reported from France[43] and Japan[44], although recent literature disputes this gene as true MODY gene. One variant was found in *HNF1B* (MODY 5) as reported to account for 2%-6% of all the diagnosed MODY cases[45]. This type was generally found to be associated with kidney dysfunction[46]. Variants in this gene have been reported from different countries[47-49]. Although it has been considered that *KLF11, BLK,* and *PAX4* are not MODY causing genes, the OMIM and recent literature reported it as involved in MODY[3,6].

Pakistan, being a developing country, is facing a huge burden of diabetes as evident from the recent survey findings that 26% of adults in the general population were suffering from diabetes[7]. There is a need to identify the genetic basis of the diabetes

in Pakistan, with large-scale efforts of screening. As Pakistan is a limited-resource society, it is important to develop sensitive and population-specific criteria. We propose our paper contributes as the first step in this direction.

A study reported that 56% of the marriages were consanguineous, and among them, over 49% were first cousin marriages[50,51], suggesting unknown recessive genes, such as seen in non-syndromic *WFS1* cases. Studies from Pakistan on MFD were very scarce. According to American Diabetes Association, the diagnosis of MFD should be considered when there is a family history of diabetes with atypical features of diabetes, such as lacking obesity[52]. There is a need to conduct large scale genetic studies on young onset diabetes to understand the genetic aspects from our country.

CONCLUSION

A wide spectrum of genetic variants involved in MFD was identified from this study. The common genes prevalent in European countries were not found common in this study. The genes other than commonly known MODY genes were identified. There is need for large scale genetic studies on early onset of diabetes in the country.

ARTICLE HIGHLIGHTS

Research background
The data on monogenic forms of diabetes (MFD) was lacking from Pakistan.

Research motivation
The identification of MFD from Pakistan will paved the way for better diagnostics and treatment for patients

Research objectives
To identify the genetic variants for MFD.

Research methods
Exome sequencing was used.

Research results
The wide spectrum of genetic variants was identified.

Research conclusions
The MODY genes prevalent in other countries, like those in Europe, were not found common in our population

Research perspectives
More studies are required, keeping in view the consanguinity rate in Pakistan

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LETTER TO THE EDITOR

Letter to editor 'Gastroenteropathy in gastric cancer patients concurrent with diabetes mellitus'

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Abstract

The present letter to the editor is related to the study titled "Diabetic gastroenteropathy: An underdiagnosed complication". Diabetic gastroenteropathy contributes to a decline in quality of life. In addition, gastroenteropathy is generally observed in patients with concurrent gastric cancer and diabetes mellitus before surgery, and the occurrence of the symptoms might be due not only to cancer but also to the complications of diabetes mellitus.

Key Words: Gastric cancer; Gastroenteropathy; Diabetes mellitus; Letter to the Editor; Commentary

Core Tip: This letter to the editor serves to analyze the relationship among gastric cancer, diabetes mellitus (DM), and gastroenteropathy, and the occurrence of the symptoms might be due not only to cancer but also to the complications of DM. In our clinical center, some of the symptoms of gastroenteropathy were in remission after gastrectomy. The reason might be radical resection of the malignant tumor. Another reason we hypothesized was DM remission after gastrectomy.

TO THE EDITOR

We read the review by Concepción Zavaleta *et al*[1] titled of "Diabetic gastroenteropathy: An underdiagnosed complication" with great interest[1]. This review systematically concluded that the pathophysiology and management of diabetic gastroenteropathy were poorly performed in patients with diabetes mellitus (DM). Although the diagnosis was not exactly accurate, after consultation and discussion in a multidisciplinary manner with experts, the core of treatment in diabetic gastroenteropathy was to delay the disease process and to restore gastrointestinal function, with blood glucose controlled by nutrition management.

We agree with the opinion in this review. It was similarly reported by previous studies that gastrointestinal discomfort in DM patients, especially gastroparesis, was associated with factors including hyperglycemia, vagal dysfunction, Cajal interstitial cells, and oxidative stress[2,3]. Gastroparesis could significantly contribute to the decline in quality of life, and nutritional status was affected by the discomfort, which included abdominal distension, vomiting, and diarrhea.

In our clinical center, we found that gastroenteropathy was generally observed in patients with concurrent gastric cancer (GC) and DM before surgery, and the occurrence of the symptoms might be due not only to cancer but also to the complications of DM. Fortunately, some of the symptoms of gastroenteropathy were in remission after gastrectomy. The reason, which could be easily estimated, was radical resection of the malignant tumor. Another reason we hypothesized was DM remission after gastrectomy, which was considered to be oncometabolism surgery in previous studies[4]. Since gastroenteropathy is considered one of the complications of DM, with the remission of DM, the symptoms of gastroenteropathy might decrease or disappear.

To our knowledge, no previous studies have analyzed the relationship among GC, DM, and gastroenteropathy. A previous study reported that the patients who underwent gastrectomy with Roux-en-Y construction showed better type 2 DM remission[5], but no study has focused on gastroenteropathy after gastrectomy. Thus, gastroenteropathy in GC patients concurrent with DM should be a focus, and a larger sample size and multicenter randomized controlled trials are needed in future studies.

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