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Contents

Contents

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Euglycemic diabetic ketoacidosis: A missed diagnosis

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Abstract

Euglycemic diabetic ketoacidosis (DKA) is an acute life-threatening metabolic emergency characterized by ketoacidosis and relatively lower blood glucose (less than 11 mmol/L). The absence of hyperglycemia is a conundrum for physicians in the emergency department and intensive care units; it may delay diagnosis and treatment causing worse outcomes. Euglycemic DKA is an uncommon diagnosis but can occur in patients with type 1 or type 2 diabetes mellitus. With the addition of sodium/ glucose cotransporter-2 inhibitors in diabetes mellitus management, euglycemic DKA incidence has increased. The other causes of euglycemic DKA include pregnancy, fasting, bariatric surgery, gastroparesis, insulin pump failure, cocaine intoxication, chronic liver disease and glycogen storage disease. The pathophysiology of euglycemic DKA involves a relative or absolute carbohydrate deficit, milder degree of insulin deficiency or resistance and increased glucagon/insulin ratio. Euglycemic DKA is a diagnosis of exclusion and should be considered in the differential diagnosis of a sick patient with a history of diabetes mellitus despite lower blood glucose or absent urine ketones. The diagnostic workup includes arterial blood gas for metabolic acidosis, serum ketones and exclusion of other causes of high anion gap metabolic acidosis. Euglycemic DKA treatment is on the same principles as for DKA with correction of dehydration, electrolytes deficit and insulin replacement. The dextrose-containing fluids should accompany intravenous insulin to correct metabolic acidosis, ketonemia and to avoid hypoglycemia.

Key Words: Diabetic Ketoacidosis; Sodium/glucose co-transporter-2 inhibitors; Pregnancy with diabetic ketoacidosis; Diabetes complications; Pregnancy in diabetes; Ketosis; Metabolic acidosis

Core Tip: Euglycemia diabetic ketoacidosis (DKA) is an uncommon, life-threatening emergency with lower normal blood glucose. Euglycemic DKA can occur in both types of diabetes mellitus, and the absence of hyperglycemia may delay diagnosis with worse outcomes. The use of sodium/glucose cotransporter-2 (SGLT-2) inhibitors as a therapeutic option in the management of diabetes mellitus has increased the incidence of euglycemic DKA. Euglycemic DKA should be considered in any unexplained metabolic acidosis with a history of diabetes mellitus and associated risk factors. Patients on SGLT-2 inhibitors must be educated about potential risk factors for euglycemic DKA and dose adjustment for sick days.

INTRODUCTION

Diabetic ketoacidosis (DKA) is widely known as a life-threatening acute complication of diabetes mellitus (DM). It mainly occurs in patients with type 1 DM; however, any acute illness like infection, trauma or acute coronary syndrome may also trigger DKA in type 2 DM. Hyperglycemia (plasma glucose > 14 mmol/L) is a hallmark in the diagnosis of DKA completing the triad with metabolic acidosis and ketonemia[1,2].

Euglycemic DKA is defined as ketoacidosis (pH < 7.3 or serum bicarbonate < 18 mmol/L) with either near-normal plasma glucose or a milder degree of hyperglycemia (11-14 mmol/L)[3,4]. The absence of hyperglycemia can conceal the underlying DKA creating a diagnostic dilemma especially in the emergency department, which is associated with worse outcomes[3-5]. Dehydration in euglycemic DKA is also minor in the absence of polyuria and polydipsia. Patients may present instead with malaise, anorexia and tachypnoea because of ketonemia and accompanying ketoacidosis. The high index of suspicion with early testing for metabolic acidosis and blood ketones can identify these patients[4].

Euglycemic DKA was first described in 1973 by Munro *et al*[6] among type 1 DM. Euglycemic DKA is an uncommon diagnosis with an incidence ranging between 2.6% to 3.2% of admissions with DKA[7,8]. In a study by Munro *et al*[6], the incidence of euglycemic DKA had an incidence of 3.2%, using a plasma glucose cut-off of less than 16.7 mmol/L[8]. However, the cut-off of plasma glucose used for euglycemic DKA in recent reviews is lower (either 14 mmol/L[3] or 11 mmol/L[4]). Authors have also used other terminology for euglycemia, like near-normal or lower than anticipated plasma glucose[9,10]. The true incidence of euglycemic DKA is therefore unknown. With the introduction of sodium/glucose cotransporter-2 (SGLT-2) inhibitors in DM management, there is a definitive increase in the published case reports or series on euglycemic DKA[9,11]. The other common causes of euglycemic DKA are pregnancy and prolonged fasting. In this review, we will discuss the pathophysiology, diagnostic considerations and management of euglycemic DKA.

PATHOPHYSIOLOGY OF EUGLYCEMIC DKA

The pathophysiology of DKA is already very well-known, characterized by a relative or absolute deficiency of insulin and excess of counterregulatory (or counter responsive) hormones like glucagon, corticosteroids, catecholamines or growth hormones[1]. The hormonal imbalance causes hyperglycemia by increasing glycogenolysis, hepatic gluconeogenesis and decreased peripheral utilization of glucose. It also promotes gluconeogenesis and ketogenesis from free fatty acid mobilization by lipolysis in adipose tissue and proteolysis of amino acids[1]. Ketone bodies (beta-hydroxybutyrate, acetoacetate and acetone) are responsible for metabolic acidosis, while hyperglycemia through glycosuria and osmotic diuresis causes dehydration and

hypovolemia (Figure 1).

Carbohydrate deficit has a pivotal role in the pathophysiology of euglycemic DKA, while insulin deficit or insulin resistance is relatively minor and secondary (Figure 1B). However, the counterregulatory hormone production is unabated, causing an increased glucagon/insulin ratio and triggering ketogenesis with no significant change in hepatic gluconeogenesis and peripheral glucose utilization[3,4,12]. The precipitating causes for euglycemic DKA include fasting or prolonged physical activity with depleted hepatic glycogen stores and hence impaired glycogenolysis[3,12]. Increased glucagon also promotes lipid oxidation, generating acetyl-CoA and ketone bodies when glycolysis intermediates are unavailable due to reduced intracellular glucose oxidation. The unabated ketonemia and glycosuria (seen usually with SGLT-2 inhibitors) contribute to euglycemic (or hypoglycemic) DKA[4,9].

The three common causes of euglycemic DKA are SGLT-2 inhibitors, pregnancy and prolonged fasting.

SGLT-2 inhibitors

SGLT-2 inhibitors are the latest group of medications added to the arsenal to treat patients with DM. Their promotion in type 2 DM is due to clinical trials suggesting protection against major adverse cardiovascular events and reduced hospitalization for heart failure and deaths[13]. SGLT-2 inhibitors have also been shown to slow chronic kidney disease progression in type 2 DM[14,15]. The added advantages appeared to be modest weight reduction and lower systolic blood pressure aside from its effect on hyperglycemia[13,15]. SGLT-2 inhibitors act by blocking the SGLT-2 cotransporter located in the early proximal renal tubule, which is responsible for the reabsorption of most (80%-90%) of the glucose filtered by the glomerulus. It leads to glucosuria and resultant lowering of blood plasma glucose concentration[16,17]. The exact mechanism that can precipitate DKA in susceptible individuals includes, osmotic diuresis along with glucosuria (causing a state of carbohydrate deficit), volume depletion and dehydration[9,16]. Carbohydrate deficit and hypovolemia promote glucagon release, increase glucagon/insulin ratio and trigger ketogenesis with euglycemia. The other factors include the direct effect of SGLT-2 inhibitors on pancreatic alpha cells, causing glucagon release and inhibiting ketone bodies excretion by the kidneys[18,19].

There has been a steady increase in the published reports on DKA with the growing use of SGLT-2 inhibitors[9]. The exact incidence rate of SGLT-2 inhibitors associated with DKA is unknown. The clinical trials of SGLT-2 inhibitors with type 2 DM have reported an incidence of 0.16 to 0.76 events per 1000 patient-years[9,20]. In a sizeable multicentric cohort study by the Canadian Network for Observational Drug Effect Studies, the incidence of DKA with SGLT-2 inhibitors in type 2 DM was 1.40 (1.29-1.53) per 1000 patient-years. The risk of DKA was nearly three-fold higher with SGLT-2 inhibitors than dipeptidyl peptidase-4 inhibitors. The increased risk of DKA was observed with all three SGLT-2 inhibitors suggesting a class effect, with canagliflozin (hazard ratio 3.58) having the highest risk[21]. In an analysis of the Food and Drug Administration's adverse event reporting system on DKA incidence with SGLT-2 inhibitors, there was a seven-fold increased risk, and around two-thirds of the reported DKA cases were euglycemic[22]. The risk is higher in patients with significant insulin insufficiency or type 1 DM (up to 9%)[9,16].

The United States Food and Drug Administration has warned against the risk of DKA with SGLT-2 inhibitors and so far has not approved its use for type 1 DM[23]. The risk of DKA with SGLT-2 inhibitors in type 1 DM varies widely across the published data of different randomized controlled trials, and factors responsible for such variation are not well understood. In a trial of dapagliflozin evaluation in patients with inadequately controlled type 1 diabetes[24], a significant number of patients in the dapagliflozin groups had DKA as compared to placebo at 52 weeks of follow-up. The risk of DKA was 4.0%, 3.4% and 1.9% in the dapagliflozin 5 mg, 10 mg and placebo groups, respectively. The DKA rate was also higher in the empagliflozin 10 mg and 25 mg groups compared with placebo in the empagliflozin as adjunctive to insulin therapy program[24]. The DKA rate was 4.3% and 3.3% with empagliflozin 25 mg and 10 mg groups, respectively, compared to 1.2% in the placebo group. It corresponds to an incidence of 5.9, 5.1 and 1.8 per 1000 patient-years[25], respectively. A similar rate of DKA has been observed in clinical trials of sotagliflozin and canagliflozin[26,27].

The duration of SGLT-2 inhibitor treatment before a diagnosis of DKA onset is hugely variable in the literature (0.3-420 days)[28]. In a recent meta-analysis by Musso *et al*[29], the risk factors of DKA with SGLT-2 inhibitors in type 1 DM included (1) baseline body mass index > 27 kg/m²; (2) insulin resistance calculated by estimated

Figure 1 Ketone bodies (beta-hydroxybutyrate, acetoacetate and acetone) are responsible for metabolic acidosis, while hyperglycemia through glycosuria and osmotic diuresis causes dehydration and hypovolemia. A: Pathophysiology of diabetic ketoacidosis; B: Pathophysiology of euglycemic diabetic ketoacidosis. FFA: Free fatty acids; ↑: Increase; ↓: Decrease; ~: No change.

glucose disposal rate < 8.3 mg/kg/min; (3) the ratio of total insulin dose reduction-to-baseline insulin sensitivity; and (4) degree of volume depletion. These risk factors should be considered by clinicians while using SGLT-2 inhibitors in type 1 DM to reduce the risk of DKA. Recently, the National Institute for Health and Care Excellence revised its guidance and recommended SGLT-2 inhibitors for the treatment of type 1 DM[30]. The patients with a body mass index of 27 kg/m^2 or more, insulin requirement of 0.5 units/kg of body weight/day or more and inadequate glycemic control despite optimal insulin therapy can be considered for the addition of dapagliflozin with insulin under supervision of a physician. However, the patient should receive education on the risk, signs and symptoms of DKA. They should also be trained on home monitoring of blood ketones and on appropriate action-plan in case of elevated blood ketones[30].

DKA in patients on SGLT-2 inhibitors can be precipitated by one of these causes (Table 1). The excessive reduction (> 50%) or omission of insulin doses, insulin pump failure or malfunction, a low carbohydrate diet, nausea and vomiting induced by other drug combination like glucagon-like peptide 1 agonists, excessive alcohol intake, acute stressful conditions like myocardial infarction, heart failure, infections or fever, trauma and surgery[9,10]. The SGLT-2 inhibitor prescription in a new-onset DM without establishing the mechanism of hyperglycemia can also precipitate DKA in undiagnosed type 1 DM[28].

Pregnancy

DKA incidence in pregnancy is significantly higher than in nonpregnant females (8.9% *vs* 3.1%) and associated with lower blood glucose levels and increased perinatal morbidity and mortality[31,32]. There are various case reports of euglycemic DKA in pregnancy with type 1 DM, type 2 DM and gestational DM[32-36]. The physiological changes of pregnancy include hypoinsulinemia and carbohydrate deficit to match the glucose requirement of the fetus and placenta[31,35,36]. The respiratory alkalosis seen with pregnancy and compensatory urinary loss of bicarbonate reduces the body reserves to buffer metabolic acidosis. There is also an insulin resistance caused by counterre-gulatory pregnancy hormones (progesterone, estrogen, human placental lactogen and tumor necrosis factor-α) seen during the second and third trimester of pregnancy. Euglycemia DKA is also common during pregnancy due to physiological hemodilution of blood glucose and increased glomerular filtration rate with

Table 1 Precipitating causes for euglycemic diabetic ketoacidosis and their mechanisms	
Risk factors	**Pathophysiology**
Infection	Insulin resistance due to counterregulatory hormones (adrenaline, glucagon, *etc*.), increased peripheral glucose utilization, decreased intake (nausea, vomiting)
Surgery	Perioperative fasting, gastrointestinal surgery has increased incidence as fasting is prolonged and/or gut absorption is slow
Fasting	Decreased glycogen stores, increased risk with SGLT-2 inhibitors and type 1 DM
Alcohol intake	Deceased carbohydrate intake, osmotic diuresis, increased ketogenesis (beta hydroxybutyrate) due to altered NADH/NAD ratio, increased risk in patients on SGLT-2 inhibitors
Acute vascular events (ACS or stroke)	Increased counterregulatory hormones, decreased oral intake
Trauma	Decreased oral intake, increased counterregulatory hormone, blood glucose dilution by large fluid shifts during resuscitation
Prolonged physical activity or exercise	Increased counterregulatory hormones, increased peripheral glucose utilization, decreased carbohydrate intake

ACS: Acute coronary syndrome; DM: Diabetes mellitus; NAD: Nicotinamide adenine dinucleotide; NADH: Nicotinamide adenine dinucleotide hydrogen; SGLT2: Sodium/glucose cotransporter-2.

glucosuria[31,35,36]. Any acute illness like infection, vomiting, fasting or short starvation can trigger ketogenesis in pregnancy. Ketogenesis and metabolic acidosis during pregnancy occur faster than when not pregnant and at lower blood sugar levels[31,35,36]. Any unexplained acidosis with a history of nausea, vomiting and decreased intake in a pregnant should raise a suspicion of euglycemic DKA[4].

Fasting
Low-calorie intake, especially with intercurrent illness in patients with type 2 DM, can precipitate DKA with euglycemia[37,38]. Patients with type 1 DM who do not adjust their insulin to low carbohydrate intake while fasting or ill can also develop euglycemic DKA[38,39]. Fasting produces a carbohydrate deficit and depletion of glycogen stores leading to alternative energy sources like free fatty acids and lipolysis[39]. Continued intake of insulin and depleted glycogen stores maintain a euglycemic state while lipolysis and ketogenesis remain unabated, triggering euglycemic DKA. A very restricted carbohydrate diet or starvation can also cause euglycemic DKA in patients without DM[4]. The fasting-induced euglycemic DKA must be differentiated from starvation ketosis in which metabolic acidosis is not present (serum bicarbonate > 18 mmol/L)[4,40]. However, euglycemic DKA during fasting or starvation is familiar with type 1 DM *vs* nondiabetic patients.

The keto diet, characterized by a low carbohydrate and high fat diet, is promoted as a popular weight-loss method and other physical or metabolic benefits[41,42]. The carbohydrate deficit and excess of fatty acids promote ketogenesis and divert ketones bodies as a source of nutrition. The weight loss is caused by reduced insulin requirement, ketone-induced osmotic diuresis and decreased oral intake because of ketonemia. The keto diet has been tried effectively in type 2 DM with weight loss benefits, better glycemic control and medication reduction for a short duration[42-45]. The benefit is found more in patients with obesity and rigorous compliance with the diet. However, the long-term effects on glycemic control, adherence and safety in patients with DM are unproven[45]. The keto diet can precipitate DKA in type 2 DM, especially during pregnancy or SGLT-2 inhibitors with a higher incidence of euglycemic DKA[44-49]. The safety of the keto diet has not been demonstrated in type 1 DM due to the risk of ketonemia and hypoglycemia[42,50].

Other causes
Euglycemic DKA has been rarely reported with other conditions like bariatric surgery[51-53], acute pancreatitis[54], sepsis[36,55], cocaine intoxication[56], insulin pump failure[56] and gastroparesis[57]. The patients undergoing bariatric surgery are prone to DKA because of perioperative deficient carbohydrate diet and prolonged fasting[4,53]. Euglycemic DKA risk is higher in type 1 DM, patients on SGLT-2 inhibitors and prolonged perioperative fasting during bariatric surgery[51,52]. Exogenous insulin administration in patients with DKA while en route to the hospital can also present lower blood glucose on admission[1].

DIAGNOSIS

Euglycemic DKA is an acute life-threatening medical emergency. The absence of hyperglycemia delays euglycemic DKA diagnosis in the emergency department or intensive care unit[3,4]. However, euglycemic DKA is a diagnosis of exclusion, and other causes of high anion gap metabolic acidosis must be excluded[3]. The common causes of high anion gap metabolic acidosis are alcoholic intoxication (excessive ethanol or toxic alcohols like methanol or polyethylene glycol), sepsis, lactic acidosis, drug overdoses (salicylate and tricyclic antidepressants) and renal failure. Other differential diagnoses include alcoholic ketoacidosis, chronic liver disease, starvation ketosis and glycogen storage disease.

Alcoholic ketoacidosis is seen in patients with chronic alcoholism[3,7]. The patient is in a state of chronic carbohydrate deficit and is dependent on alcohol for calories. Any acute illness that can cause an inability to consume alcohol triggers ketonemia and ketoacidosis. The presentation is similar to euglycemic DKA with gastrointestinal symptoms (nausea, vomiting or abdominal pain), metabolic acidosis and ketonemia. Some authors consider alcoholic ketoacidosis as a subtype of euglycemic DKA[3,7]. The pathophysiology is also similar with an increased glucagon/insulin ratio. However, a history of binge alcohol consumption, nondiabetic and hypoglycemia instead of euglycemia helps diagnose alcoholic ketoacidosis[58]. The ketone bodies in alcoholic ketoacidosis are predominantly β-hydroxybutyrate (instead of acetoacetate), which could not be detected on routine urine strip testing[59]. Serum ketones must be used for detection of ketonemia in the cases of suspicion of alcoholic ketoacidosis.

Euglycemic ketoacidosis because of either fasting or any intercurrent illness with reduced calorie intake needs to be differentiated from starvation ketosis[4,40]. No previous history of DM, no intercurrent illness and hypoglycemia differentiate starvation ketosis. The metabolic acidosis is also not profound, with bicarbonate levels usually more than 18 mmol/L.

Sepsis[36,55] with or without associated lactic acidosis is a common presentation in an emergency that can conceal euglycemic DKA. High lactate levels with the absence of serum ketones help in the diagnosis of sepsis.

Unexplained high anion gap metabolic acidosis in a patient with DM and associated risk factors should raise suspicion of euglycemic DKA. A detailed history of risk factors like pregnancy, surgery, fasting, infections and SGLT-2 inhibitors should be evaluated (Table 1). The laboratory tests include serum and urine ketones, electrolytes (including calcium and magnesium), glucose, renal function (creatinine, blood urea nitrogen), blood gas analysis (venous or arterial), lactic acid, chest radiograph and electrocardiogram. Wide osmolar gap (the difference between measured and calculated serum osmolarity), inebriate state and multiorgan involvement help to diagnose toxic alcohol ingestion. History and symptoms of infection with laboratory tests showing leukocytosis, procalcitonin, organ dysfunction and lactate help diagnose sepsis and septic shock.

TREATMENT

The treatment is straightforward once the diagnosis is made. The treatment is based on the same principles as in DKA[1]: Insulin to correct metabolic acidosis and anion gap and correction of electrolytes and dehydration. The fluid resuscitation is similar to DKA with correction of dehydration and starts with balanced crystalloids. Insulin replacement using a fixed rate intravenous insulin infusion calculated on 0.1 units/per kilogram body weight should be continued until anion gap (metabolic acidosis) correction, and the patient can accept orally. However, an early glucose requirement (for prevention of hypoglycemia) allows concomitant insulin infusion to suppress ketogenesis[3]. Dextrose (10% or 5%) and intravenous infusion of insulin must be used until the anion gap and metabolic acidosis is corrected. The resolution of DKA is defined as pH > 7.3 units, bicarbonate > 15.0 mmol/L and blood ketone level < 0.6 mmol/L[1]. Patients may require intensive care unit admission and monitoring for hemodynamic and electrolyte disturbances. The laboratory monitoring for acidosis, glucose and electrolytes must be similar to DKA management.

PREVENTION OF EUGLYCEMIC DKA

The patients who are prescribed SGLT-2 inhibitors should be explained the risk factors of DKA (Table 1). The off-label use of SGLT-2 inhibitors in type 1 DM should be done with close monitoring, starting with a lower dose, personalized insulin reduction regimen and patient education on carbohydrate intake[9,10]. Patient education on "sick days" and other lifestyle modifications are essential and should be done in patients with type 1 and type 2 DM[9]. Education about stopping SGLT-2 inhibitors when feeling ill or feverish, prolonged exercise, fasting or excessive alcohol intake must be done. The drug should also be stopped 3-4 days before planned surgery and adjust the insulin regimen accordingly[9,10].

CONCLUSION

Euglycemic DKA can be a missed diagnosis in the emergency department with worse outcomes. The presence of metabolic acidosis in a patient with DM and risk factors should be assessed for ketonemia, even in the absence of hyperglycemia. Patients on SGLT-2 inhibitors must be educated about risk factors and dose adjustment for sick days.

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New insights into renal lipid dysmetabolism in diabetic kidney disease

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Conflict-of-interest statement:
Alessia Fornoni and Sandra Merscher are investors on pending or issued patents (US 10183038 and US 10052345) aimed at diagnosing or treating proteinuric kidney diseases. They stand to gain royalties from the future commercialization of these patents. Alessia Fornoni is Vice-President of L&F Health LLC and is consultant for ZyVersa Therapeutics, Inc. ZyVersa

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Abstract

Lipid dysmetabolism is one of the main features of diabetes mellitus and manifests by dyslipidemia as well as the ectopic accumulation of lipids in various tissues and organs, including the kidney. Research suggests that impaired cholesterol metabolism, increased lipid uptake or synthesis, increased fatty acid oxidation, lipid droplet accumulation and an imbalance in biologically active sphingolipids (such as ceramide, ceramide-1-phosphate and sphingosine-1-phosphate) contribute to the development of diabetic kidney disease (DKD). Currently, the literature suggests that both quality and quantity of lipids are associated with DKD and contribute to increased reactive oxygen species production, oxidative stress, inflammation, or cell death. Therefore, control of renal lipid dysmetabolism is a very important therapeutic goal, which needs to be archived. This article will review some of the recent advances leading to a better understanding of the mechanisms of dyslipidemia and the role of particular lipids and sphingolipids in DKD.

Key Words: Diabetes; Lipids; Free fatty acids; ATP-binding cassette transporters sub-class A; Sterol-O-acyltransferase 1; CD36; Sphingolipids; Sphingomyelin phosphodiesterase acid-like 3b; Diabetic kidney disease

Core Tip: The present review summarizes the recent knowledge about the role of lipids and sphingolipids in the development and progression of diabetic kidney disease (DKD). The main focus is given to the cholesterol and triglyceride metabolism abnormalities, lipid droplet accumulation and role of sphingolipids in DKD.

INTRODUCTION

Lipids are essential components of a cell plasma membrane with multiple cellular functions, highlighting their importance in cell homeostasis and survival. Diabetic kidney disease (DKD) is often considered to be a consequence of hyperglycemia in a setting of diabetes mellitus. However, lipid accumulation in podocytes, which are specialized epithelial cells lining the urinary surface of the glomerular capillary tuft, has been recently reported to drive the development of DKD[1]. Lipids are also key modulators of insulin signaling in several cell types including the podocyte[2,3].

The toxicity of lipid accumulation (lipotoxicity) in the kidney was first proposed by Moorhead *et al*[4] in 1982 and later updated by Ruan *et al*[5] in 2009, suggesting that lipid dysmetabolism promotes the progression of kidney diseases, including DKD. However, the specific contribution of podocyte lipid dysmetabolism to the pathogenesis and progression of DKD has been largely unexplored. Growing evidence suggests that lipotoxicity-associated renal damage depends not only on the quantity of lipids that accumulate in the kidney but also on the lipid species[6]. In recent years, a clear role of sphingolipids and glycolipids in the pathogenesis of DKD has been also established[7-11]. Given the fact that podocytes, the terminally differentiated epithelial cells in the glomerulus, are main contributors to the proper filtration function in the kidney, changes in their number[12] and function lead to the development and progression of glomerular disease, including DKD. However, what is the cause of podocyte detachment and death in DKD remains largely unknown. We have previously published several reviews related to the role of lipids and sphingolipids in glomerular diseases with focus on insulin signaling[2], inflammation[13], and mitochondria dysfunction[14]. This review is an update on the latest knowledge with regard to the mechanisms contributing to renal lipid dysmetabolism focusing on cholesterol metabolism, fatty acid oxidation, lipid droplet accumulation and sphingolipids and how they contribute to the development and progression of DKD.

CHOLESTEROL METABOLISM ABNORMALITIES IN DKD

In any cell, lipid metabolism encompasses the synthesis and degradation of lipids to meet the body's energy needs. Some lipids are being constantly oxidized, while others are being synthesized and stored. Thus, triacylglycerols are broken into free fatty acids (FFA), which undergo β-oxidation in mitochondria to produce acetyl coenzyme A (CoA), utilized in the tricarboxylic acid cycle or ketogenesis to generate energy. FFA are also involved into other biosynthetic pathways to produce membrane lipids (such as phospholipids, glycolipids, sphingolipids, or cholesterol) or signaling molecules (such as prostaglandins, leukotrienes, and thromboxanes). These metabolic pathways are tightly regulated by enzyme-catalyzed reactions and defects in any of these enzymes is associated with a wide range of health problems.

Podocytes are visceral epithelial cells of the glomerulus, which are involved in filtration and formation of primary urine. Foot processes are the most recognizable characteristic structures of podocytes and the formation of specialized junctions between foot process of neighboring podocytes, known as the slit diaphragm, and of foot processes and the glomerular basement membrane, known as the adhesion

complex, are important for maintaining glomerular function[15]. The podocyte slit diaphragm is assembled in lipid rafts, which are small specialized plasma membrane domains enriched with cholesterol, sphingolipids and protein complexes with important functions in cellular signaling transduction. Cholesterol of the lipid rafts plays an important role in regulating the organization, localization and function of proteins within the slit diaphragm. Excess of cholesterol negatively affects the binding of slit diaphragm proteins to each other[16], or interferes with the ability of podocyte slit-diaphragm proteins to bind caveolin-1, an important transductor of the insulin receptor signaling in podocytes[17].

Cholesterol is synthesized starting from acetyl CoA in the *de novo* pathway and/or it can be imported from circulating lipoproteins by receptor-mediated endocytosis (influx). Excess cholesterol is released through several distinct pathways (efflux). Tight regulation of these three mechanisms is very important to maintain proper cholesterol metabolism within the cell, as unesterified (free) cholesterol is toxic to cells.

Cholesterol synthesis

Intracellular cholesterol sensing is mainly regulated *via* sterol regulatory element-binding protein (SREBP, and its known isoforms SREBP-1a, SREBP-1c, SREBP-2), an endoplasmic reticulum resident. Increased expression of SREBP1 and SREBP2 has also been reported in glomeruli of DKD patients based on microarray data available from the Nephroseq database[18,19]. Increased expression of SREBP has been described to contribute to kidney damage in obesity-related diabetes and in mice fed on a high fat diet[20-24]. Additional studies demonstrated a role of SREBP1 in the accumulation of lipid droplet in murine models of type 1 diabetes[25]. In support, the inhibition of SREBP isoforms was found to attenuate the renal phenotype such as albuminuria or mesangial expansion in age-related renal disease and in DKD[16-20]. In contrast, a recent study reports that fatostatin treatment of 12-wk-old male mice with strepto-zotocin-induced diabetes, an inhibitor of SREBP-1 and SREBP-2, prevents glomerular basement membrane thickening, but does not improve albuminuria or hyperfiltration[26]. Thus, further studies are needed to determine if SREBP inhibition may be more beneficial in combination with other therapies to prevent DKD progression.

Cholesterol influx

Cholesterol is transported in the circulation by two major lipoproteins, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The influx of cholesterol is primarily mediated *via* LDL receptors (LDLR), followed by endocytosis and the formation of LDL-containing vesicles connected to lysosomes. Free cholesterol is then transported to the endoplasmic reticulum (ER) or plasma membrane *via* Niemann Pick C1 or C2 transporters. In the ER, increased free cholesterol levels activate sterol-O-acyltransferase 1 (SOAT1; or acyl-CoA:cholesterol acyltransferase (ACAT1)) to form cholesterol esters for storage in lipid droplet. We recently demonstrated that genetic loss of SOAT1 in diabetic *db/db* mice ameliorates kidney injury by reducing cholesterol esters and lipid droplet accumulation[27]. More recently, proprotein convertase subtilisin/Kexin Type 9 (PCSK9) inhibitors, which have been developed to controlled hyperlipidemia by affecting LDL uptake and clearance in hepatocytes, have been shown to control the hyperlipidemia associated with nephrotic syndrome[28]. As PCSK9 is also expressed in the kidney[29], the contribution of PCSK9 to renal lipotoxicity remains to be explored.

Cholesterol efflux

Excessive cholesterol accumulation in podocytes is also associated with suppressed efflux in both experimental[22,30] and human DKD[6]. Cholesterol efflux from cells, including podocytes, occurs primarily *via* ATP-binding cassette transporters sub-class A (ABCA1), G (ABCG1) and scavenger receptor class B type I (SR-BI). We previously reported that normal human podocytes exposed to serum from patients with type 1 and type 2 diabetes and early stage of DKD are characterized by increased lipid droplet accumulation and reduced expression of ABCA1[3,31,32]. We also found that the expression of ABCA1 correlates with markers of DKD progression clinically and in experimental mouse models (diabetic BTBR *ob/ob* and *db/db* mice)[32]. Studies in diabetic NOD mice also demonstrated significant reduction (48%) of ABCA1 expression in kidneys[30]. While deficiency of ABCA1 is a susceptibility factor in DKD and contributes to the accumulation of lipid droplet in podocytes, it is not sufficient to cause glomerular injury itself[31,32]. Further studies demonstrated that ABCA1 overexpression reduces albuminuria in mice with podocyte-specific activation of

nuclear factor of activated T cells (NFAT)[31], another susceptibility factor for cholesterol-dependent podocyte injury. Interestingly, in human glomerular cells, interleukin 1β has also been shown to inhibit cholesterol efflux possibly *via* suppression of ABCA1 expression[33]. By contrast, pharmacological induction of cholesterol efflux using cyclodextrin or ezetimibe, a small molecule ABCA1 inducer, resulted in amelioration of DKD progression and DKD-like glomerulosclerosis[3,32]. Exendin-4, an agonist of glucagon-like peptide 1, has also been shown to upregulate ABCA1 in glomerular endothelial cells and improve glomerular hypertrophy, basement membrane thickening and mesangial expansion[34]. Interestingly, in diabetic patients (*n* = 1746, all Caucasians), the ABCA1 rs9282541 (R230) polymorphism has been shown to be associated with increased risk of diabetes, while the ABCA1 rs1800977 (C69T) polymorphism was found to be associated with a significantly reduced risk of hypertriglyceridemia[35]. The rs9282541 polymorphism has also been reported to be associated with susceptibility to type 2 diabetes in patients from Mexico[36]. In contrast, studies in patients with type 2 diabetes (*n* = 107) from Turkey[37] and in Chinese Han population (*n* = 508)[38] failed to link ABCA1 rs1800977 polymorphism to lipid dysmetabolism. More recently, an association between LXR-alpha and ABCA1 gene polymorphisms was found to be associated with the risk for DKD in a Chinese population[39]. While ABCA1 mediates cholesterol transport to apolipoprotein A-I (Apo A-I) and pre-β HDL, two other transporters, ABCG1 and SR-BI, mediate cholesterol transport to mature HDL. In mouse models of DKD, significant suppression of ABCG1 and SR-BI was found in mesangial and tubular cells[40].

Taken together, these studies demonstrate that cholesterol accumulation and lipid droplet accumulation may represent a hallmark of DKD[41-43]. Based on our own studies and reports from others, we conclude that cholesterol accumulation in glomerular cells occurs independent of systemic cholesterol levels and that local lipid dysmetabolism contributes to DKD progression in patients with diabetes (Figure 1).

TRIGLYCERIDE METABOLISM ABNORMALITIES IN DKD

Fatty acid uptake
In the blood most of the circulating lipids are present as triglycerides within very low-density lipoprotein (VLDL). Triglycerides are composed of free fatty acid (FFA) and glycerol. Several fatty acid transport proteins (FATPs) control uptake of FFA into a cell. In the kidney, FATP1, FATP2, and FATP4 were shown to be mostly responsible for lipid uptake abnormalities in patients with DKD. Thus, a recent study on a population of type 2 diabetic patients (*n* = 268) demonstrated that expression levels of FATP1 and FATP2 in plasma are associated with progression of DKD[44]. In support, deletion of FATP2 in different mouse models of DKD (*db/db* and *eNOS-/-* diabetic mice and low dose streptozotocin-induced diabetic mice on a high fat diet) was sufficient to improve the renal outcome[45]. It has been also demonstrated that expression of FATP4 is higher in tubules of mice on a high fat diet[46], suggesting a role of FATP4 in insulin resistance and obesity. Interestingly, levels of FATP4 in *db/db* mice were shown to be elevated in parallel with increased renal lipid accumulation and progression of DKD, which is also associated with vascular endothelial growth factor B (VEGF-B) signaling[1]. In obese Wistar rats on high fat diet increased levels of FFA in glomerular endothelial cells were shown to be associated with microalbuminuria *via* VEGF-NO axis[47]. In patients with type 2 diabetes FATP4 is associated with glomerular filtration rate[48].

Other contributors to the lipid uptake abnormalities in DKD are the fatty-acid binding proteins (FABPs), which belong to a super-family of lipid-binding proteins and recognize long-chain fatty acids as substrates. Thus, urinary liver-type FABP (L-FABP) was shown to be a reliable marker of DKD development and progression in patients with diabetes[49-52]. Interestingly, in spontaneously diabetic Torii fatty rats higher levels of urinary L-FABP were shown, which was ameliorated with Liraglutide treatment[53].

Fatty acid uptake: role of CD36
Cluster of differentiation 36 (CD36), a class B scavenger receptor, is the most important transmembrane glycoprotein that mediates uptake of oxidized LDLs. CD36 is also the main uptake system of FFA in the kidney, where it is highly expressed in proximal and distal epithelial cells, podocytes and mesangial cells. Increased expression of CD36 seems to be associated with kidney damage in DKD. Earlier studies demonstrated that

Figure 1 Cellular dyslipidemia in diabetic kidney disease affects cholesterol synthesis, influx and efflux. Sterol regulatory element-binding protein 1 or 2 (SREBP1 and SREBP2) is transported from the endoplasmic reticulum to the Golgi apparatus, where it is cleaved followed by translocation to the nucleus to initiate cholesterol synthesis. Newly synthesized free cholesterol is then converted into esterified cholesterol by sterol O-acyltransferase 1 (SOAT1) or is transported to the plasma membrane for efflux by ATP-binding cassette subfamily A member 1 (ABCA1), subfamily G member 1 (ABCG1) or scavenger receptor class B type I (SR-BI). Cholesterol uptake from circulating low density lipoproteins (LDLs) is mediated by LDL receptor (LDLR). In diabetic kidney disease (DKD), overexpression of SREBP1 and SREBP2 and decreased expression of ABCA1, ABCG1 and SR-BI results in accumulation of cholesterol inside a cell and increased reactive oxygen species production. Accumulation of free cholesterol activates SOAT1, leading to over-production of esterified cholesterol, which is toxic to cells. Overexpression of proprotein convertase subtilisin kexin 9 may also contribute to DKD *via* enhanced degradation of the LDLR, resulting in increased levels of circulating LDL cholesterol. This image was created using BioRender software (www.BioRender.com). SREBP1: Sterol regulatory element-binding protein 1; SREBP2: Sterol regulatory element-binding protein 2; ER: Endoplasmic reticulum; SOAT1: Sterol O-acyltransferase 1; NCEH: Neutral cholesterol ester hydrolase; LDLox: Oxidized low density lipoprotein; PCSK9: Proprotein convertase subtilisin kexin 9; LDLR: Low density lipoprotein receptor; ROS: Reactive oxygen species; LCAT: Lecithin:cholesterol acyltransferase; APOL1: Apolipoprotein L1; APOE: Apolipoprotein E; APOM: Apolipoprotein M; APOA1: Apolipoprotein A1; HDL: High density lipoprotein; ABCA1: ATP-binding cassette subfamily A member 1; ABCG1: Subfamily G member 1; SR-BI: Scavenger receptor class B type I; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; Pi: Inorganic phosphorus.

high glucose-mediated overexpression of CD36 induces apoptosis in renal tubular epithelial cells[54,55] and podocytes[56]. Interestingly, CD36 has also been shown to facilitate chronic inflammation, oxidation stress and fibrosis in proximal tubular cells under hyperglycemic conditions[57]. Using human podocytes, our studies suggest a novel mechanism where discoidin domain receptor 1 (DDR1), a tyrosine kinase activated by collagen I, interacts with CD36 and leads to increased CD36-dependent FFA uptake[58]. Another study demonstrated that astragaloside IV inhibits overexpression of CD36 in human glomerular mesangial cells and diabetic rats (Sprague Dawley) in response to palmitate-induced FFA accumulation and attenuates FFA uptake, oxidative stress and fibrosis[59].

In mouse podocytes treated with palmitic acid increased expression of CD36 has been shown in association with increased reactive oxygen species (ROS) production and apoptosis[60]. In mice with transgenic overexpression of CD36 in the kidney, accumulation of lipids and triglycerides in kidneys was demonstrated[61]. Additionally, CD36 is involved in the generation of other cell-specific responses *via* toll-like receptors (TLRs) 2, 4 and 6[62-64], CD9[65], or integrin[66] leading to the activation of pyrin domain-containing 3 (NLRP3) and nuclear factor kappa B (NF-kB) signaling pathways[67,68]. Indeed, CD36 can also recognize advanced oxidation protein products and advanced glycation end products, which are also involved in inflammatory pathway activation[69], including the kidney[57].

In patients with DKD increased expression of CD36 was reported[55,60]. Interestingly, a circulating soluble form of CD36 (sCD36), whose derivation is not entirely clear, may play a role as a cellular source of CD36 in diabetic patients and correlates with insulin resistance[70,71]. A recent study demonstrated elevated levels of sCD36 in both plasma and urine of patients with DKD[72]. However, while one study suggests that sCD36 Levels are elevated in patients with type 2 diabetes and proposes to use it as a biomarker[73], another study reports no differences in the sCD36 Levels between patients with type 1 and type 2 diabetes[74].

Thus, CD36 has an important role in the lipid homeostasis in the kidney with an important role in the crosstalk between CD36 Ligands and inflammation or apoptosis signaling pathways. Therefore, CD36 may represent a promising target for therapeutic intervention. However, further studies of the role of CD36 in DKD progression are needed to answer the questions: (1) How is sCD36 formed in patients with diabetes and what is the tissue-specific role of sCD36? (2) What are the particular mechanisms of increased FFA uptake in tubular cells *vs* podocytes? And (3) What are the mechanisms involved in the kidney cell-specific regulation of CD36 levels or function (tubular cells *vs* podocytes)? A better understanding of the mechanisms regulating the FFA uptake in rodents and its translation to humans will be a determinative factor in the development of novel peptides aimed at regulating CD36 Levels with minimum off-target effects.

Fatty acid oxidation

Fatty acid oxidation (FAO), also called beta oxidation, is the aerobic process of fatty acid (short-, medium- or long-chain saturated fatty acyl coenzyme A, acyl-CoA) breakdown that occurs in mitochondria to provide energy from fats. During FAO, acetyl coenzyme A (acetyl-CoA), five molecules of ATP, and water are generated. Interestingly, FAO covers more than half of renal oxygen consumption. In the setting of kidney disease, genes that are associated with FAO are significantly downregulated in kidneys of mice and humans[61], which is also associated with increased fatty acid synthesis and higher intracellular lipid deposition. We have recently reported that human podocytes cultured in the presence of serum from DKD patients have significantly decreased expression of FAO genes (PPARα, ACADM, ACOX1/2), which was also observed in mouse models of DKD and in our mouse model of ABCA1 deficiency[32]. In a longitudinal study on American Indians with type 2 diabetes (*n* = 92), a significant reduction of FAO has been shown, which was also associated with lower abundance of C16-C20 acylcarnitines[75]. Pharmacological or genetic increase in FAO has been shown to be beneficial to improve kidney disease progression[61].

Peroxisome proliferator-activated receptors (PPARs) play a key role in the regulation of FAO in the kidney. PPARg, one of the PPARs isoforms, is highly expressed in different compartments of a nephron while decreased expression contributes to diabetes-associated kidney damage. Activation of PPARg (using thiazolidinediones) is associated with attenuation of kidney function in diabetic patients and mouse models of DKD[76-78]. Recently, a role of micro-RNA-27a (miR-27a) in the regulation of PPARg activity was demonstrated[79], suggesting miR-27a as a potential therapeutic target in DKD. In a streptozotocin-induced diabetic mouse model of DKD, activation of PPARδ ameliorates diabetes-associated renal damage [80]. Lack of PPARα, another PPAR isoform, has also been shown to accelerate DKD in a streptozotocin-induced diabetic mouse model[81]. Tesaglitazar, the PPARα/g dual agonist, markedly attenuated albuminuria and lowered collagen deposition in kidneys of db/db mice[82]. In contrast, use of a PPARα agonist, CP-900691, showed no effect on albuminuria and amelioration of DKD in the *BTBR ob/ob* diabetic mouse model[83]. Therefore, while activation of PPARg seems to have constitutive renoprotective effects in DKD, the role of PPARα activation in improving renal function remains questionable. A summary of the suggested mechanism of triglyceride abnormalities in DKD is shown in Figure 2.

SPHINGOLIPIDS IN DKD

Sphingolipids are important components of cell homeostasis. Sphingolipids are a class of lipids composed of hydrophobic and hydrophilic regions with variable fatty acid composition. In recent years, sphingolipids and sphingolipid metabolites have been recognized as important regulators of cell signaling contributing to the development and progression of numerous diseases. The most studied sphingolipid metabolites are ceramide, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), which have been shown to regulate cell differentiation, membrane fluidity, protein anchoring, immune activation, insulin sensitivity, autophagy, and cell death. The role of S1P signaling in renal cells and in kidney diseases has been extensively reviewed[84].

Ceramide

In kidney cortices of diabetic *db/db* mice, elevated levels of long-chain ceramides (C14:0, C16:0, C18:0, C20:0) and decreased levels of very-long-chain ceramides (C24:0,

Figure 2 Abnormalities in triglyceride homeostasis contribute to lipid droplet accumulation in diabetic kidney disease. Increased expression of scavenger receptor class B (CD36), fatty acid transporter protein 1 (FATP1), FATP2, FATP4 and fatty acid-binding protein leads to accumulation of fatty acids inside a cell, abnormalities in triglyceride (TG) metabolism and formation of TG-enriched lipid droplets. Altered activity of perilipin protein family members (PLIN2 and possibly PLIN5) also contributes to lipid droplet formation. In turn, accumulation of TG-enriched lipid droplet causes alteration in oxidative phosphorylation, cardiolipin accumulation and reactive oxygen species overproduction. Together with increased expression of NLR family pyrin domain containing 3 and interleukin 1β CD36 overexpression causes podocyte injury in diabetic kidney disease. This image was created using BioRender software (www.BioRender.com). FFA: Free fatty acid; DDR1: Discoidin domain receptor 1; FATP1: Fatty acid transporter protein 1; FATP2: Fatty acid transporter protein 2; FATP4: Fatty acid transporter protein 4; FABP: Fatty acid transporter protein; NLRP3: NLR family pyrin domain containing 3; PLIN2: Perilipin protein family member 2; PLIN5: Perilipin protein family member 5; TG: Triglyceride; OXPHOS: Oxidative phosphorylation; ROS: Reactive oxygen species; IL-1β: Interleukin 1 beta.

C24:1) have been described[85], which is in accordance with our own studies[7]. In support of previous studies, ceramide accumulation was associated with increased reactive oxygen species production in OLEFT rats and in mice fed on a high-fat diet with DKD[86]. Elevated levels of long-chain ceramides (C16:0, C18:0 and C20:0)[87,88] and very-long-chain ceramides (C22:0, C24:0)[88] were also found in patients with early or overt DKD. Podocyte-specific deletion of the acid ceramidase main catalytic subunit (*Asah1* gene) results in elevated ceramide levels in glomeruli and development of nephrotic syndrome in mice[89]. In patients with DKD enrolled into ONTARGET and TRANSCEND-randomized controlled trials rs267734 gene variant of ceramide synthase 2 (CerS2), a CerS2 isoform with high expression in the kidney, has been shown to be associated with increased albuminuria[90].

Sphingosine-1-phosphate

In the setting of diabetes, increased levels of S1P in plasma of rodents with type 1[91] or type 2 diabetes[92] have been reported. In mice with streptozotocin-induced diabetes increased renal levels of S1P were also reported[93,94]. Recent studies in mice and humans demonstrated that mutations in *SGPL1* gene, which encodes S1P lyase 1, are associated with the development of nephrotic syndrome[9,10,95]. In rats with streptozotocin-induced DKD the use of an unselective S1P receptor agonist (FTY720) was found to have a renoprotective effect[96]. Interestingly, plasma levels of S1P in patients with type 2 diabetes negatively correlate with levels of albuminuria, while less S1P is observed in patients with macroalbuminuria[97]. A role of S1P lyase activity reduction has been demonstrated to contribute to the development of podocyte-based kidney toxicity in wildtype rodents[11]. Furthermore, S1P receptor signaling plays a significant role in glomerular injury. Five S1P receptors (S1PR1-S1PR5) exist, of which S1PR1 to S1PR4, but not S1PR5, are expressed in the kidney[98]. In mouse models of DKD, activation of S1PR1 or inhibition of S1PR2 prevented the renal injury

phenotype[96]. Using a single cell RNA sequencing approach to profile glomerular cells in mouse models of DKD (streptozotocin-induced diabetic endothelial nitric oxide synthase-deficient mice), significantly lowered expression of S1P receptor 3 (S1PR3) in mesangial cells was demonstrated[99]. Previous studies also revealed a significant role of sphingosine kinase (SPHK), an enzyme that generates S1P from sphingosine, in the kidney fibrosis in STZ-induced diabetic mice and in humans with DKD[100]. In a mouse model of alloxan-induced diabetes, increased glomerular SPHK1 expression and activity were demonstrated leading to S1P accumulation[101]. In addition, SPHK1 upregulation was demonstrated in STZ-induced mouse model of DKD, where it protects from the fibrotic process[100]. A more detailed review on the role of S1P signaling in the kidney was previously published by us[84].

Ceramide-1-phosphate

Even less is known about the role of C1P in the kidney. In contrast to S1P, C1P is most likely released from damaged cells[102]. Our studies demonstrated that increased sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b) in the *db/db* mouse model of DKD is associated with a state of C1P deficiency in podocytes[7]. SMPDL3b is a lipid-raft associated protein[103] that regulates plasma membrane fluidity[104] by blocking access of ceramide kinase, an enzyme that generates C1P from ceramide, to ceramide[8]. We also reported that elevated expression of SMPDL3b occurs in glomeruli of patients with DKD[105] and that SMPDL3b overexpression in podocytes results in the accumulation of S1P[106]. In support, podocyte-specific deficiency of *Smpdl3b* resulted in restoration of the renal C1P content in association with delayed DKD progression in diabetic mice[7]. To the contrary, others demonstrated that the knockout of ceramide kinase in mice is sufficient to prevent glomerular disease[107]. However, it remains to be established how bioactive sphingolipids contribute to the development of DKD and what are the best options for their use as possible biomarkers or therapeutic targets.

Glycosphingolipids

Dysmetabolism of other sphingolipids, such as gangliosides (mainly GM3, which is the most abundant ganglioside in the kidney), has also been reported to contribute to development of DKD[108]. Increased levels of sialic acid, a component of gangliosides, were found in patients with DKD and positively correlated with blood glucose, HbA1c, creatinine and microalbuminuria[109]. Increased GM3 species (C16:0, C18:0, C20:0, C22:0, C24:0) in kidney cortex from diabetic rats at an early stage of DKD have also been described[110]. Interestingly, GM3 was found to contribute to diabetic nephropathy *via* the alteration of pro-survival receptor-associated Akt signaling[111]. Another study reported that levels of glycosylated sphingolipids, such as lactosyl-ceramide, are associated with microalbuminuria in patients with type 1 diabetes[112]. A proposed mechanism indicating how dysregulation of sphingolipid metabolism contributes to DKD is shown in Figure 3.

LIPID DROPLET ACCUMULATION IN DKD

Lipid droplet (or lipid bodies) are lipid-rich cellular organelles that regulate storage and hydrolysis of lipids or serve as a reservoir for cholesterol and acyl-glycerol in different eukaryotic cells. Structurally, lipid droplets are composed of a neutral lipid core (triacylglycerol and cholesteryl esters) and a phospholipid monolayer. In an eukaryotic cell, lipid droplet formation may be induced by different stimuli, such as growth factors, long-chain unsaturated fatty acids, oxidative stress and inflammatory stimuli (reviewed in Ref.[113]). Once intracellular, the fatty acids can form part of the triglyceride and phospholipid components of the lipid droplet[114,115]. Increased lipid droplet accumulation is observed in patients with DKD[6] and mouse models of DKD[116,117]. We previously showed that treatment of human podocytes with serum from patients with DKD results in lipid droplet accumulation[3]. Kidneys of hyperglycemic mice (STZ-induced diabetes) are characterized by the concomitant presence of oxidative stress markers-positive (xanthine oxidoreductase and nitrotyrosine with tail-interacting protein of 47 kDa) lipid droplets in glomerular and/or tubular cells[117]. In Sprague-Dawley rats with STZ-induced diabetes, increased advanced glycation end products have been shown to cause lipid droplet accumulation[118].

Figure 3 Dysregulation of sphingolipid metabolism contributes to the progression of diabetic kidney disease. Decreased activity of desaturase (DEGS2) results in the accumulation of dihydroceramides. Increased activity of ceramide synthase 2 (CERS2) leads to increased production of ceramide (Cer), which leads to increased production of sphingosine (Sph) and sphingosine-1-phosphate (S1P) *via* decreased activity of sphingosine-1-phosphate lyase 1. Cer can also be translocated to the Golgi apparatus *via* ceramide transport protein, where it results in production of sphingomyelin. At the plasma membrane, decreased activity of sphingomyelin phosphodiesterase 2 affects Cer production, while elevated activity of alkaline ceramidase 2 increases levels of Sph, which, in turn, leads to accumulation of S1P *via* increased activity of sphingosine kinase 1. Overproduction of S1P results in increased S1P efflux *via* S1P transporters (such as ATP-binding cassette transporters ABCA1, ABCG1, ABCC1 and S1P transporter SPNS2), where S1P can act as a paracrine factor to activate S1P receptor signaling (primarily, S1P receptors 1-3, S1PR1, S1PR2, S1PR3), leading to dysregulation of many cellular processes, including migration, proliferation, survival or inflammation. Accumulation of gangliosides (GM3) can also affect cell survival in diabetic kidney disease. This image was created using BioRender software (www.BioRender.com). SMPD2: Sphingomyelin phosphodiesterase 2; SM: Sphingomyelin; C1P: Ceramide-1-phosphate; CERK: Ceramide kinase; SMPDL3b: Sphingomyelin phosphodiesterase acid-like 3b; Cer: Ceramide; Sph: Sphingosine; S1P: Sphingosine-1-phosphate; S1PR1: Sphingosine-1-phosphate receptor 1; S1PR2: Sphingosine-1-phosphate receptor 2; S1PR3: Sphingosine-1-phosphate receptor 3; GM3: Ganglioside M3; CERT: Ceramide transport protein; SGPL1: Sphingosine-1-phosphate lyase 1; DH-Cer: Dihydroceramide; ASAH1: N-acylsphingosine amidohydrolase 1; CERS2: Ceramide synthase 2; DEGS2: Delta(4)-desaturase, sphingolipid 2; SPTLC2: Serine palmitoyltransferase 2; CoA: Acyl-coenzyme A; ROS: Reactive oxygen species.

While the composition of lipid droplets is not very well investigated, perilipins are the best characterized proteins of the lipid droplet coat. This family of perilipin proteins includes perilipin 1 (PLIN1), perilipin 2 (PLIN 2), perilipin 3 (PLIN 3), perilipin 4 (PLIN 4) perilipin 5 (PLIN 5). Not much data about the role of these proteins in DKD development and progression, and a recent case report suggests that mutation in *PLIN1* may be associated with DKD-like kidney damage in a patient with type 4 familial partial lipodystrophy[119]. Another randomized case-control study of an Iranian population (*n* = 200) showed an association of the polymorphism rs4578621 in the *PLIN* gene with type 2 diabetes[120]. Interestingly, decreased *Plin1* expression was reported in adipocytes of *db/db* mice, while deficiency of *Plin1* in adipose tissue in wildtype mice resulted in insulin resistance and secretion of pro-inflammatory lipid metabolites, such as prostaglandins[121]. Expression of *PLIN2* is significantly upregulated in kidneys from diabetic *db/db* mice[122] and in podocytes of patients with DKD[1], which may indicate that increased PLIN2 expression may contribute to increased lipid droplet accumulation in the diabetic kidney. Similarly, increased levels of urinary *PLIN2* were reported in patients with DKD[123]. To date, no studies examining the role of other perilipin proteins in DKD have been performed. A role for *PLIN5* in diabetes has recently been suggested as upregulation of *PLIN5* in β-cells was shown to improve glucose tolerance in isolated islets from mice or human[124]. Because *PLIN5* is also expressed in kidneys[125] under PPAR control, it would be important to investigate its role in lipid-associated kidney diseases in future investigations.

Among other factors contributing to lipid droplet accumulation in the kidney, autophagy has been shown to regulate lipid metabolism and lipid droplet formation[126-128] and to significantly contribute to renal fibrosis progression in kidney diseases. Serine/threonine protein kinase 25 (STK25), which plays an important role in skeletal muscle metabolism, is also highly expressed in human and rodent kidney[129] and was shown to aggravate renal lipid accumulation and exacerbate kidney injury in a high-fat diet mouse model of DKD[130].

CONCLUSION

The kidney is a target organ of the harmful effects of lipotoxicity in diabetes, suggesting that, similar to the liver, chronic kidney disease is a form of fatty kidney disease. In this review, we summarize new research trends and new scientific knowledge acquired within the past few years that have shed light on the role of particular lipids in diabetes-associated kidney injury. However, our knowledge with regard to the cross-talk between glucose homeostasis and lipid metabolism in health and disease remains incompletely understood and further research is needed. Similarly, more insight into the role of specific lipids in podocyte physiology is required to answer remaining questions. Which lipids are toxic to the podocytes? What factors are driving the pathophysiology of lipid accumulation in podocytes? Which lipids might be the best targets for possible therapeutic intervention in DKD? Answering these questions will help to pave the way to new diagnostic and therapeutic approaches in DKD.

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Recent advances in new-onset diabetes mellitus after kidney transplantation

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Abstract

A common challenge in managing kidney transplant recipients (KTR) is post-transplant diabetes mellitus (PTDM) or diabetes mellitus (DM) newly diagnosed after transplantation, in addition to known pre-existing DM. PTDM is an important risk factor for post-transplant cardiovascular (CV) disease, which adversely affects patient survival and quality of life. CV disease in KTR may manifest as ischemic heart disease, heart failure, and/or left ventricular hypertrophy. Available therapies for PTDM include most agents currently used to treat type 2 diabetes. More recently, the use of sodium glucose co-transporter 2 inhibitors (SGLT2i), glucagon-like peptide-1 receptor agonists (GLP-1 RA), and dipeptidyl peptidase 4 inhibitors (DPP4i) has cautiously extended to KTR with PTDM, even though KTR are typically excluded from large general population clinical trials. Initial evidence from observational studies seems to indicate that SGLT2i, GLP-1 RA, and DPP4i may be safe and effective for glycemic control in KTR, but their benefit in reducing CV events in this otherwise high-risk population remains unproven. These newer drugs must still be used with care due to the increased propensity of KTR for intravascular volume depletion and acute kidney injury due to diarrhea and their single-kidney status, pre-existing burden of peripheral vascular disease, urinary tract infections due to immunosuppression and a surgically altered urinary tract, erythrocytosis from calcineurin inhibitors, and reduced kidney function from acute or chronic rejection.

Key Words: Cardiovascular disease; Glucagon-like peptide-1 receptor agonists; Kidney transplantation; Oral antihyperglycemic drugs; Post-transplant diabetes mellitus; Sodium glucose co-transporter 2 inhibitors; Dipeptidyl peptidase-4 inhibitors

Core Tip: Kidney transplant recipients commonly develop post-transplant diabetes mellitus. Sodium glucose co-transporter 2 inhibitors, glucagon-like peptide-1 receptor agonists, and dipeptidyl peptidase 4 inhibitors are now available for treating type 2 diabetes mellitus. There is increasing evidence that these classes of drugs are effective in kidney transplant recipients, but caution is still advised due to their increased propensity otherwise for intravascular volume depletion, infections, and reduced kidney function.

INTRODUCTION

Kidney transplantation (KT) is the renal replacement therapy of choice in patients with end-stage kidney disease (ESKD), improving quality of life and reducing mortality risk compared to dialysis[1]. However, an adverse effect of KT is post-transplant diabetes mellitus (PTDM). PTDM adversely affects patient survival and quality of life[2,3], leading to greater risk of graft loss, rejection, and infection, as well as diabetes-associated microvascular and macrovascular complications[4]. Graft failure for example is 50% higher in kidney transplant recipients (KTR) with diabetes than without diabetes, and recurrent diabetic kidney disease occurs in almost half of kidney allografts[5,6]. About one-third of nondiabetic KTR develop persistently impaired glucose metabolism by 6 mo post-transplantation[7-9]. Risk factors for PTDM include older recipient age, deceased donor graft, the use of calcineurin inhibitors (CNI) and corticosteroids, and adult polycystic kidney disease, in addition to traditional risks factors for type 2 diabetes (T2DM).

PTDM describes newly diagnosed T2DM after organ transplantation, regardless of timing or undetected pre-transplant presence, and is applied to clinically stable patients with persistent post-transplantation hyperglycemia[10]. Therefore, PTDM is often formally diagnosed at least 45 d post-transplant due to the high prevalence of early post-transplant hyperglycemia. The term PTDM now excludes known pre-existing diabetes mellitus (DM). Common measures to combat PTDM include early treatment with insulin, lifestyle interventions such as diet and exercise, bariatric surgery, and modified immunosuppression such as CNI and steroid avoidance. Since treatment approaches to pre-existing T2DM and PTDM do not significantly differ, the discussion of PTDM is taken throughout this review to encompass pre-existing DM.

Comprehensive reviews of PTDM have been published[11]. More recently, sodium glucose co-transporter 2 inhibitors (SGLT2i), glucagon-like peptide-1 receptor agonists (GLP-1 RA), and dipeptidyl peptidase 4 inhibitors (DPP4i) are becoming increasingly available for managing T2DM. This update reviews the role of these newer agents in managing PTDM.

Current management of PTDM

At the 2013 international consensus meeting on PTDM, committee members were unable to endorse a hierarchical approach to using antihyperglycemic agents for managing PTDM. Suggestions included altering the immunosuppressive regimen and starting antihyperglycemic agents on an individualized basis[10]. CNI and steroid doses are often reduced, dietary counseling provided, and oral agents started. Despite the plethora of pharmacotherapy options for treating T2DM and by extension PTDM, there is paucity of evidence on the efficacy and safety of SGLT2i, GLP-1 RA, and DPP4i in KTR. In addition to healthy behavior interventions, metformin remains first line therapy in T2DM-associated chronic kidney disease (CKD) as well as PTDM[12-14]. Most recently, the Kidney Disease: Improving Global Outcomes (KDIGO) 2020 guidelines recommend metformin plus SGLT2i as first-line, followed by any other antihyperglycemic agent with GLP-1 RA being preferred as second line[15]. However, the safety and efficacy of SGLT2i when estimated glomerular filtration rate (eGFR) is < 30 mL/min per 1.73 m^2 in KTR is limited, but further studies will clarify their kidney and cardiovascular (CV) benefits[15].

Connecting PTDM to the cardiorenal syndrome

Managing PTDM connects to managing other facets of the cardiorenal syndrome, and as will be discussed in subsequent sections, can involve using SGLT2i, GLP-1 RA, and DPP4i. CV disease (CVD) leads causes of death in KTR, accounting for 30% of all deaths with a functioning graft[16,17]. KTR also carry a burden of other CV risk factors including hypertension, dyslipidemia, and obesity, all exacerbated by immuno-suppressive medications[18]. KTR risk higher mortality than their age-matched counterparts without kidney disease[19]. This mortality risk is almost two-fold greater in PTDM[20]. For KTR with pre-existing diabetes, the risk of CVD and stroke increases threefold compared to non-diabetic recipients[21].

The most common CVD in KTR is ischemic heart disease (IHD), congestive heart failure (CHF) and left ventricular hypertrophy (LVH). IHD contributes over 50% to mortality in KTR[21]. CHF occurs 2-5 times more in KTR than the general population[22], reaching almost 20% by 3 years post-KT[23]. DM can cause heart failure (HF) independently of IHD *via* a diabetic cardiomyopathy with either preserved or reduced ejection fraction (HFpEF, HFrEF). HF is 2- to 4-fold more prevalent in DM and occurs earlier[24]. Diabetic nephropathy influences drug dosing in HF, resulting in treatment adjustments and failure to attain therapeutic targets. Risk factors for new-onset HF post-KT include DM[22,23,25]. LVH, a risk factor for sudden cardiac death in KTR, occurs in 50%-70% of this population. In non-KTR with T2DM, large CV and renal outcome trials of SGLT2i and GLP-1 RA have shown that these medications are safe, improve glycemia, and carry CV and renal benefits[26].

SGLT2I

SGLT2i act selectively on the sodium-glucose 2 cotransporter in the proximal tubule of the nephron that reabsorbs approximately 90% of filtered glucose, to effectively prevent its reuptake and promote its urinary excretion to reduce blood levels. Glycosuria results whenever filtered glucose exceeds the maximum absorption rate by SGLT2 co-transporters. SGLT2i reduce hemoglobin A1c (HbA1c) by 0.5%-0.7% in an insulin-independent manner with minimal risk of hypoglycemia, leading to weight loss[26]. SGLT2i cause an osmotic diuretic and natriuretic effect that leads to plasma volume contraction, in turn decreasing systolic and diastolic blood pressure (BP) by 4-6 and 1-2 mmHg, respectively[27]. Since filtered glucose load depends on blood glucose, SGLT2i achieve their greatest blood glucose reduction during hyperglycemia. Glucose-lowering efficacy declines from reduced glycosuria as GFR declines. SGLT2i-induced natriuresis leads to increased sodium delivery to the macula densa, and tubular glomerular feedback results in afferent arteriolar vasoconstriction, with reduced intraglomerular hypertension, GFR and albuminuria. Natriuresis-related reductions in BP and possibly renoprotection persist even with reduced kidney function[28]. It should be remembered that KTR still have CKD; the eGFR is often 50 mL/min per 1.73 m^2 or less, and CKD associates with CVD. Therefore, the hypothesis that SGLT2i reduce CV risk in KTR is worth exploring.

SGLT2i are available both individually and combined with metformin and DPP4i. Sotagliflozin is a dual SGLT2/1i for treating both T2DM and T1DM. Sotagliflozin also inhibits intestinal SGLT2, delaying glucose absorption and post prandial glucose rise[29].

Adverse effects of SGLT2i

SGLT2i cause mycotic genital or yeast infections, often with candida species, in about 9%-18% of women with half this rate in men[30-32]. Urinary tract infections (UTI) are less common. Euglycemic diabetic ketoacidosis (DKA), while rare, occurs in the context of insulin deficiency, sudden reductions in insulin dose, or increased dose requirements from illness, surgery or alcohol abuse[12]. The incidence of DKA was increased with dapagliflozin[33], while increased lower limb amputations were seen with canagliflozin[32]. However, a meta-analysis of randomized clinical trials (RCT) found no class effect-based increased risk for amputation[34]. Volume depletion may worsen perfusion of an already dysfunctional vascular network, but this hypothesis remains unproven[35]. Fracture risk may be higher with canaglifozin but this risk was unconfirmed by meta-analysis[36]. SGLT2i may also affect bone metabolism and density[37].

SGLT2i and CV protection

SGLT2i reduce 3-point major adverse CV events [MACE: Death from CV causes, non-fatal myocardial infarction (MI) or non-fatal stroke], all-cause mortality and HF hospit-alizations in the general population in varying combinations[31-33]. SGLT2i sig-nificantly reduced MACE in those with established CVD[38]. Potential beneficial mechanisms include naturietic diuresis, reduced inflammation, and increased hematocrit from erythropoietin production with enhanced myocardial tissue oxygen delivery[39].

Several trials specifically examined HF as a primary outcome[38,40-42]. Many patients did not have T2DM, and SGLT2i reduced CV death and HF hospitalization or progression regardless of diabetes status[43,44]. Patients with HFrEF of < 40% showed a significantly lower CV death or HF hospitalization again regardless of T2DM status[44], and a slower eGFR decline in T2DM[45]. With T2DM and recent worsening HF there was lower CV mortality and HF hospitalization.

LVH has not been studied to the same extent as CV mortality and HF. However, a substudy of the EMPA-HEART (Effects of Empagliflozin on Cardiac Structure in Patients With Type 2 Diabetes) CardioLink-6 RCT showed that empagliflozin was associated with significant reduction in LV mass index, possibly from increased red cell mass and improved myocardial tissue oxygen delivery[46].

SGLT2i and kidney protection

Empagliflozin was associated with slower CKD progression, reduced albuminuria progression, and reduced ESKD or death and maintenance[45]. The CANVAS trial using canagliflozin showed a reduced eGFR decline and reduced albuminuria in T2DM[47], while CREDENCE demonstrated both reduced kidney failure and CV events in T2DM[48]. The DAPA-CKD trial of dapagliflozin in CKD with or without T2DM demonstrated a lower composite of sustained decline in eGFR by 50%, ESKD, or death from renal or CV causes[49]. A systematic review and meta-analysis of data from EMPA-REG, CANVAS, CREDENCE, and DECLARE TIMI 58 found that SGLT2i reduced risk of dialysis, acute kidney injury (AKI), and death due to kidney disease in patients with T2DM eGFR levels down to 30 mL/min per 1.73 m²[50].

A pre-specified meta- analysis of trials involving empagliflozin and dapagliflozin on hospitalisations for HF were consistent, suggesting that they improve renal outcomes, all-cause and CV death in patients with HFrEF[51]. Another meta-analysis showed that SGLT2i improved CV and kidney outcomes, regardless of T2DM, HF, and/or CKD status, with the greatest benefit for HF-related hospitalization and CKD progression[52].

SGLT2i use in KTR with PTDM

KTR are typically excluded from large clinical trials, including registration trials. The safety and efficacy of SGLT2i in non-KT patients with T2DM is now well-established, and so has led to attempts to extend the study of SGLT2i to KTR. A recent systematic review and meta-analysis of 8 studies in 132 KTR showed that SGLT2i were effective in lowering HbA1C and body weight, and preserved kidney function with no serious adverse events such as euglycemic ketoacidosis or acute rejection[53]. Fourteen patients had a UTI, one patient had a myocotic gential infection, one AKI, and one cellulitis. Another recent review concluded that SGLT2i are safe, along with GLP-1 RA and DPP4i, but are not as efficacious as in non-diabetic non-KTR[54].

A small RCT using empagliflozin in 22 KTR (versus 22 placebo) showed that the magnitude of HbA1c reduction depended on eGFR and baseline HbA1c, with no significant difference in adverse events, immunosuppressive drug levels, or eGFR[55]. A pilot study to replace insulin with empagliflozin in 14 stable KTR resulted in weight loss, but also significant drop-out and increased HbA1c, necessitating the reinstitution of insulin therapy in some[56]. SGLT2i were not as efficacious in KTR compared to other diabetic groups, perhaps from lower eGFR and the vasoconstrictive effect of CNI. A case series of 10 KTR demonstrated that the median HbA1c decreased from 7.3% to 7.1%[57]. An uncontrolled study of canagliflozin in 24 KTR, 23 of who were male, showed reduced body weight, BP, HbA1c, and need for other hypoglycemic agents. There were also no hypoglycemic episodes[58]. Other small series have reported similar findings[59]. Another experience using canagliflozin of 10 patients that also included 4 simultaneous pancreas-KT recipients showed that the magnitude of improvements in glycemic control, weight, and BP are similar to nontransplant patients[60]. A search of the Cochrane Kidney and Transplant Register of Studies reported that SGLT2i probably do not affect kidney graft survival compared to placebo, but may improve glycemic control without causing hypoglycemia and

affecting eGFR long-term[61].

Erythrocytosis has been noted with SGLT2i[62]. A well-described adverse event seen in KTR is post-transplant erythrocytosis[63]. Increased erythropoietin production is seen with SGLT2i and may be beneficial in the general population, but whether this is a positive effect in KTR is unknown. Posttransplant erythrocytosis (PTE) occurs in 8%-15% of KTR and affects patients with well-preserved graft function, commonly 8-24 mo post-KT[64]. PTE can cause malaise, headache, lethargy, dizziness, thromboembolic events, and a 1%-2% mortality. Endogenous erythropoietin may play a central role in PTE with a defect in normal feedback regulation, and persistent erythropoietin secretion from native kidneys. PTE is generally treated with angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, and occasionally phlebotomy[64,65]. A randomized study of empagliflozin for 6 mo in non-KT patients suggested that hematocrit increase is at least partly the result of increased erythropoietin and stimulated erythropoiesis rather than hemoconcentration, based on changes in red blood cell (RBC) morphology, reduced ferritin or iron stores, differential time course of response, and reduced RBC hemoglobin concentration[46]. This safety concern regarding erythrocytosis particularly needs evaluation in KTR.

Dose adjustments of SGLT2i in CKD are recommended (Table 1). Since glucose-mediated hyperfiltration and high blood sugar levels combine to increase glucose filtration[66], the glucose-lowering effects of SGLT2i are decreased at an eGFR < 60 mL/min and almost absent with eGFR < 30 mL/min.

KDIGO guidelines do not currently recommend using SGLT2i in KTR[15]. When used, however, proper patient education is key in reducing the risk of SGLT2i-related complications. Patients should be counseled on sick day management that includes temporarily stopping SGLT2i during periods of illness with vomiting, diarrhea or other states that risk dehydration or intravascular volume depletion. as well as around planned surgical procedures. These illnesses are common early after KT. The eGFR is in a state of flux, with frequent change in kidney function from acute rejection, infections, and CNI-induced vasoconstriction. CNI dose adjustments are frequent. Transplant renal artery stenosis may occur from edema at the anastomotic site, and there is often surgical manipulation of the urinary tract, including stent removal. KTR may also have a pre-existing burden of peripheral vascular disease or recurrent UTI. Peripheral arterial disease may be worsened due to the anastomosis of the kidney allograft to the external iliac artery, further reducing lower extremity arterial perfusion. Peri-transplant ischemic CV events may occur, further affecting effective circulating volume. Post-KT hyperglycemia also commonly improves with routine corticosteroid dose reduction in the first few months after KT, and so new antihyperglycemic medication introduction is often delayed. Diuretics are commonly prescribed early after KT for volume expanded states, especially if heart function is reduced or there is significant peripheral edema. Some patients experience difficulty in unlearning the salt-restricted diet imposed by dialysis. SGLT2 inhibition is natriuretic[66] and may theoretically potentiate the natriuretic action of prescribed diuretics.

In summary, despite the prevalence of meticulously studied endpoints in large clinical trials in the general population, as well as small clinical trials and observational studies in KTR, it remains unclear if the cardiorenal benefits associated with SGLT2i in the general population (with or without DM) will more generally translate to PTDM. There is also presently no reason to use SGLT2i in non-diabetic KTR. Nonetheless, SGLT2i appear to be well-tolerated, but should preferably be avoided in the early post-surgical phase of KT.

GLP-1 RA

The incretin system has become an essential target for managing T2DM. Incretins are hormones produced by the intestinal mucosa in response to oral food intake, and enhance insulin while suppressing glucagon secretion in a glucose dependent manner to lower blood glucose[67-70]. Thus, incretins reduce insulin release when glucose levels are near-normal. Incretin hormones include glucose-dependent insulinotropic polypeptide (GIP) and GLP-1. GLP-1 also slows gastric emptying and increases satiety, leading in-turn to weight loss. Insulin secretion is greater in response to oral than intravenous glucose intake, the so-called "the incretin effect", but this effect decreases in T2DM[70]. The GLP-1 effect declines with impaired insulin secretion, insulin resistance, and hyperglycemia, all leading to decrease in GLP-1 receptor expression and increased GLP-1 resistance[70-72]. GLP-1 RA are eliminated by proteolytic degradation and glomerular filtration, so their metabolism does not involve CYP- or

Table 1 Newer antihyperglycemic agents and chronic kidney disease

CKD stage	1	2	3a	3b	4	5
eGFR (mL/min per 1.73 m^2)	≥ 90	60-89	45-59	30-44	15-29	≤ 15
SGLT2 inhibitors						
Canagliflozin (Invokana)	300 mg OD	Dose adjustment not required	Reduce dose to 100 mg OD if < 60 mL/min	Reduce dose to 100 mg OD in previously treated patients with albuminuria > 33.9 mg/mol. Do not initiate if < 30 mL/min		
Dapagliflozin (Forxiga)	10 mg OD	Dose adjustment not required		Not recommended	Contraindicated	
Empagliflozin (Jardiance)	25 mg OD	Dose adjustment not required			Contraindicated	
Ertugliflozin (Steglatro)	15 mg OD	Dose adjustment not required	Not recommended for initiation of therapy. Discontinue if persistently < 45 mL/min		Contraindicated	
Sotogliflozin (Zynquista)	400 mg OD	Dose adjustment not required	Not recommended for initiation of therapy. Discontinue if persistently < 45 mL/min		Contraindicated; safety not established	
GLP-1R agonists						
Dulaglutide (Trulicity)	1.5 mg weekly	Dose adjustment not required			Caution as safety not established	
Exenatide (Byetta)	10 μg BID	Dose adjustment not required	Caution if 30-50 ml/min		Not recommended due to risk of accumulation	
Liraglutide (Victoza)	1.8 mg OD	Dose adjustment not required			Safety not established	
Lixisenatide (Adlyxine)	20 μg OD	Dose adjustment not required		Safety not established		
Semaglutide (Ozempic)	1 mg weekly	Dose adjustment not required			Limited experience	Not recommended
Semaglutide (Rybelsus)	14 mg OD	Dose adjustment not required			Limited experience	Not recommended
DPP4 inhibitors						
Alogliptin (Nesina)	25 mg OD	Dose adjustment not required	Reduce dose to 12.5 mg		Reduce dose to 6.25 mg	
Linagliptin (Trajenta)	5 mg OD	Dose adjustment not required				Limited experience
Saxagliptin (Onglyza)	5 mg OD	Dose adjustment not required	Reduce dose to 2.5 mg if < 50 mL/min			Not recommended
Sitagliptin (Januvia)	100 mg OD	Dose adjustment not required	Reduce dose to 50 mg if < 50 mL/min		Reduce dose to 25 mg	
Vildaglitin (Galvus)	50 mg BID	Dose adjustment not required	Reduce dose to 50 mg OD if < 50 mL/min			

CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; SGLT2: Sodium glucose co-transporter 2; GLP-1R: Glucagon-like peptide-1 receptor; DPP4: Dipeptidyl peptidase 4.

transported-mediated drug-drug interactions. GLP-1 RA are incretin mimetics.

Hypoglycemia may occur if GLP-1 RA is given concomitantly with an insulin secretagogue. Most GLP-1 RA are administered subcutaneously, but there is one oral GLP-1 RA available (Table 1). Common adverse effects include nausea, vomiting, diarrhea, and injection-site reactions. GLP-1 RA are contraindicated for patients with a history of medullary thyroid cancer, multiple endocrine neoplasia 2, or pancreatitis. Oral intake must be adequate for GLP-1 RA to be given.

GLP-1 RA, CV, and kidney protection

GLP-1 RA studies were generally conducted in individuals with established atherosclerotic CVD. Except lixisenatide[72], all current GLP-1 RA are associated with a reduction in risk of MACE in patients with T2DM and established CVD. Lixisenatide, liraglutide, and dulaglutide all demonstrated CV safety[72-75].

The LEADER trial using liraglutide that included individuals with eGFR 15-30 mL/min per 1.73 m², demonstrated a greater benefit to MACE reduction with eGFR < 60 mL/min[73]. Liraglutide added to standard care resulted in lower new-onset and slower progression of diabetic CKD, driven primarily by persistent macroalbuminuria, with a similar rate of renal adverse events including AKI to placebo. The REWIND trial using dulaglutide showed, besides reduced MACE, a reduction in new severely increased albuminuria, sustained eGFR decline of 30% from baseline, or renal replacement therapy[76]. The AWARD 7 trial of once-weekly dulaglutide in moderate-to-severe CKD produced glycaemic control similar to insulin, with reduced eGFR decline[77]. SUSTAIN 6 CVOT using weekly semaglutide also demonstrated safety and significantly reduced MACE in posthoc analysis for superiority[78]. A systematic review and meta-analysis showed that GLP-1 RA are cardioprotective across many population subgroups, and reduce HF hospitalization and all-cause mortality[79]. In summary therefore, besides CVD risk reduction with GLP-1 RA, there is also risk reduction in new-onset albuminuria, eGFR decline, and progression to ESKD or kidney death.

GLP-1 RA use in KTR with PTDM

GLP-1 RA are recommended by most guidelines as second line as an alternate to an SGLT2i after metformin in managing T2DM especially with CVD, CV risk factors, or CKD. Small case series using GLP-1 RA in KTR do exist, showing no serious adverse effects or immunosuppressive drug interactions[80]. However, the evidence for use in KTR remains very limited. A review of the Cochrane Kidney and Transplant Register found no randomized, quasi-RCT and cross-over studies examining the effects of GLP-1 RA on safety and efficacy for treating pre-existing and new onset diabetes in KTR[61].

The rationale for using GLP-1 RA in KTR is that incretin therapies are able to counterbalance the interference of immunosuppressive drugs on insulin secretion. Corticosteroids are commonly used in anti-rejection regimens for KTR along with CNI (tacrolimus and cyclosporine), all of which affect glucose metabolism by decreasing glucose utilization and enhancing hepatic gluconeogenesis. Corticosteroids also directly decrease insulin secretion and increase insulin resistance. CNI impair α-cell and β-cell function and the incretin effect. The mechanism of action of GLP-1 RA may be ideal in this situation due to their insulinotropic, glucagonostatic and glucose-lowering effects that directly target defects linked to immunosuppressive-induced hyperglycemia[81], although drug interactions such as CNI resulting in increased drug exposure remain a concern[82]. Weight loss is another benefit of GLP-1 RA since weight gain is a common consequence of both hyperglycemia and KT more generally, making GLP-1 RA especially appealing for PTDM.

A study examining the role of hyperglucagonemia in PTDM, and the insulinotropic and glucagonostatic effects of GLP-1 during fasting and hyperglycemic states, concluded that PTDM is characterized by reduced glucose-induced insulin secretion and attenuated glucagon suppression. Moreover, similar to T2DM, GLP-1 infusion reduced glucagon concentration and increased first- and second-phase insulin secretion[82]. A major concern of GLP-1 RA in KTR is delayed gastric emptying, potentially affecting absorption of co-administered narrow therapeutic index medications such as CNI[83]. Although GLP-1 RA are not metabolized by the liver or involved in cytochrome or transporter mediated drug-drug interaction, there may be a delay in drug concentration, but it appears drug exposure may not be affected. Thus GLP-1 RA are theoretically safe, but close monitoring of tacrolimus and cyclosporine concentrations and potential side effects is required. A case series on safety of coadministration of liraglutide and tacrolimus found that tacrolimus AUC_{0-12h} reduced but trough levels were not affected[80], and there was no evidence of acute rejection.

A chart review of KTR who received liraglutide for glycemic control showed significant improvement in A1C, FBS, eGFR and body weight with minimal side effects[84]. Another retrospective study that included 7 KTR with PTDM receiving GLP-1 RA for 12 mo found no significant changes in tacrolimus concentration or kidney function[85]. A large experience of 63 KTR with PTDM using dulaglutide found sustained reduction in body mass index and insulin requirement for up to 24 mo, without increased risk of cancer, CV events, graft-failure, or all-cause mortality. Gastrointestinal side effects were infrequent and there was no requirement for change in immunosuppressive therapy[86]. A recent study however did not demonstrate weight loss, but did show reduced total daily insulin dose and a low risk of hypoglycemia with no adverse effect on kidney allograft outcomes[87].

DPP4I

DPP4i, otherwise known as gliptins, prevent the inactivation of GLP-1 and GIP. They are once daily drugs with the exception of vildagliptin[88]. Higher levels of endogenous GLP-1 enhance incretin action including glucose-dependent insulin secretion. They slow gastric emptying, increase satiety, and reduce postprandial glucagon secretion. DPP4i are generally well tolerated, have a low risk for hypoglycemia, and are weight neutral, but can cause acute pancreatitis[88].

DPP4i, CV, and kidney protection

All major CV trials of DPP4i including linagliptin[89], sitagliptin[90], saxagliptin[91], and alogliptin[92] revealed non-inferiority compared to placebo for the risk of major events. Non-inferiority was also evident when linagliptin was compared to glimepiride[93]. However, in the SAVOR-TIMI 53 trial, saxaliptin was associated with an increased risk of hospitalization for HF in patients with elevated N-terminal pro B-type natriuretic peptide levels, a history of HF, or CKD with eGFR < 60 mL/min[94]. Linaglipitin and saxagliptin reduce the risk for albuminuria progression, or even improve albuminuria, regardless of baseline eGFR[95,96]. This benefit was not demonstrated with sitagliptin[97]. The KDIGO 2020 guidelines highlight the role of DPP4i in T2DM and CKD. Therefore, while DPP4i may be useful adjuncts to control blood glucose and favorably affect albuminuria at best, their effect on CVD outcomes and CKD progression remains uncertain.

DPP4i use in KTR with PTDM

Most diabetes practice guidelines such as those of Diabetes Canada and KDIGO recommend DPP4i as add-on therapy for patients without CVD in whom glycemic targets are not achieved, especially if a lower risk of hypoglycemia and/or weight gain are priorities. A systematic review and meta-analysis of 5 studies in KTR with PTDM found that DPP4i improved glycemic control compared to either placebo or other oral anti-hyperglycemic agents, but did not significantly affect eGFR or tacrolimus concentration[98]. A meta-analysis including eight DPP4i studies showed both efficacy and safety[99]. A search of the Cochrane Kidney and Transplant Register[61] described the evidence concerning DPP4i as being of low to very low certainty. A study of 65 KTR demonstrated increased cyclosporine concentrations with sitagliptin but not linagliptin[100].

CONCLUSION

Safety data for SGLT2i, GLP-1 RA, and DPP4i are reassuring, and the CV and kidney risk reduction benefits are certainly substantial for SGLT2i and GLP-1 RA in non-KTR with T2DM. GLP-1 RA do not share benefits similar to SGLT2i with respect to preventing HF. GLP-1 RA are a potential treatment option for PTDM to help offset the increased CV risk associated with KT. Incretin therapy uniquely counteracts the interference of immunosuppressants on insulin secretion. DPP4i are useful for glycemic control. The first priority in managing KTR is achieving glycemic control; any CV and kidney benefits should be considered incidental at this time.

More RCT are needed to support using all three drugs in KTR. The UTI risk with SGLT2i may be especially concerning for KTR. With a single kidney, volume sensitivity may theoretically risk AKI, and so sick day management education is critical. SGLT2i, GLP-1 RA, and DPP4i may eventually prove to be ideal choices for both glycemic control and cardiorenal protection in KTR, but the evidence in KTR for now remains limited. The risk of intravascular volume depletion, brought on by the use of diuretics, renal artery stenosis, and diarrhea due to mycophenolic acid may compound the concern for AKI. Sick day management of other drugs is already prescribed to KTR. Dialysis patients also need to unlearn their salt restricted diet. Other potential concerns in KTR include worsening post-transplant osteoporosis, with most bone loss occurring early after KT. KTR may also carry a burden of peripheral vascular disease, occasionally worsened by the anastomosis of the kidney allograft to the external iliac arterial system. The hemoglobin should be monitored. These special considerations are described in Table 2. However, there is no reason that any of the newer antihyperglycemic drugs cannot be used in KTR as long as patients are carefully monitored. The early studies involving KTR are all generally favorable.

Table 2 Special considerations in prescribing newer antihyperglycemic agents to kidney transplant recipients

Clinical evidence	Largely observational
Kidney function	Reduced glomerular filtration rate
	Fluctuating glomerular filtration rate
	Post-transplant diuresis
Surgically altered urinary tract	Urinary tract infections
Graft arterial anastomosis	Peripheral vascular disease
Immunosuppression	Fluctuating glucose control
	Interaction with calcineurin inhibitors
	Urinary tract infections
Gastrointestinal upset	Intravascular volume depletion
	Dehydration
Others	Post-transplant erythrocytosis

In summary, initial evidence seems to indicate that newer antihyperglycemic agents can be used in KTR. It may be preferable to avoid these drugs in the first 6 mo after KT due to the increased frequency of infections typically seen from enhanced immunosuppression coupled with an anatomically altered urinary tract, as well as susceptibility to intravascular volume depletion and the volume sensitivity of a solitary kidney. These drugs should not be considered first-line agents, but can be prescribed cautiously in the context of poor glycemic control after other suitable measures specific to KTR have already been undertaken.

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Renal gluconeogenesis in insulin resistance: A culprit for hyperglycemia in diabetes

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Abstract

Renal gluconeogenesis is one of the major pathways for endogenous glucose production. Impairment in this process may contribute to hyperglycemia in cases with insulin resistance and diabetes. We reviewed pertinent studies to elucidate the role of renal gluconeogenesis regulation in insulin resistance and diabetes. A consensus on the suppressive effect of insulin on kidney gluconeogenesis has started to build up. Insulin-resistant models exhibit reduced insulin receptor (IR) expression and/or post-receptor signaling in their kidney tissue. Reduced IR expression or post-receptor signaling can cause impairment in insulin's action on kidneys, which may increase renal gluconeogenesis in the state of insulin resistance. It is now established that the kidney contributes up to 20% of all glucose production *via* gluconeogenesis in the post-absorptive phase. However, the rate of renal glucose release excessively increases in diabetes. The rise in renal glucose release in diabetes may contribute to fasting hyperglycemia and increased postprandial glucose levels. Enhanced glucose release by the kidneys and renal expression of the gluconeogenic-enzyme in diabetic rodents and humans further point towards the significance of renal gluconeogenesis. Overall, the available literature suggests that impairment in renal gluconeogenesis in an insulin-resistant state may contribute to hyperglycemia in type 2 diabetes.

Key Words: Renal gluconeogenesis; Insulin-resistance; Insulin; Insulin receptor signaling; Diabetes; Gluconeogenic enzymes

Core Tip: Recently, investigators have begun elucidating the role of renal gluconeogenesis in physiology and pathology. Recent evidence suggests a significant role of the kidney in glucose metabolism under pathological conditions, such as insulin resistance

and diabetes. This review summarizes the findings from the literature that have enhanced our knowledge related to the significance of renal gluconeogenesis in normal and pathological states.

INTRODUCTION

Gluconeogenesis is the process of glucose production by non-carbohydrate carbon substrates. During the process, glucose-6-phosphate is produced from precursors, like lactate, glycerol, and amino acids, with subsequent hydrolysis by glucose-6-phosphatase (G6Pase) to glucose. Previously, kidney was not considered to significantly contribute to the overall glucose release[1], however, re-evaluation using the net balance techniques suggested up to 20% contribution to overall glucose production[2]. The rate of renal gluconeogenesis varies in response to physiological activities, such as fasting, postprandial, exercise, stress, and pathological stimuli, like diabetes and insulin sensitivity[3-5].

The liver, kidney, and intestine are the three tissues that express the key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and G6Pase. G6Pase helps in the final release of glucose into the circulation by dephosphorylating glucose-6-phosphate. PEPCK is involved in the phosphorylation of oxaloacetic acid and FBPase dephosphorylates fructose-1,6 bisphosphate to fructose-6-phosphate. The activity of these enzymes is regulated by insulin. Besides, insulin also regulates the other rate-limiting step, like the availability of gluconeogenesis substrates[6-8]. Renal gluconeogenesis is more sensitive to insulin activity than hepatic gluconeogenesis[3]. Impaired insulin action due to inefficient receptor expression/signaling may blunt insulin's suppressive effect on gluconeogenesis. It could contribute to hyperglycemia as seen in insulin-resistant and diabetic rat models and humans[9-15]. Patients with type-2 diabetes mellitus exhibit an increase of about 300% in glucose production[16,17]. Glucose-induced glucose release by the kidneys may potentially contribute to postprandial hyperglycemia in diabetic patients[3]. Renal gluconeogenesis contributes to normal glucose levels in the post-absorptive state and plays a key role in postprandial hyperglycemia in diabetic patients[5].

GLUCOSE PRODUCTION AND UTILIZATION BY THE KIDNEYS

The kidneys' substantial contribution to systemic glucose levels *via* gluconeogenesis has now been recognized[18-20]. The first evidence of glucose release by the kidneys emerged in 1938 when Bergman *et al*[21] reported doubled glucose utilization in the hepatectomized animals along with nephrectomy. Several studies confirmed that renal cortex can produce glucose from non-carbohydrate precursors[9,22-25]. The primary sources for renal glucose production involve lactate from cellular respiration, glutamine from protein, and glycerol from triglyceride breakdown[26]. Other than the *in vitro* studies, incorporating these precursors into glucose by the human kidney has also been quantitated[27,28]. Studies using the isotopic approach in human subjects suggested lactate to be the most important renal gluconeogenic substrate, followed by glutamine and glycerol[3,28,29]. Several studies have suggested kidney's role in maintaining glucose homeostasis through gluconeogenesis[18,19,26]. Early human studies using a combination of net renal glucose balance and isotopic measurements have demonstrated that the kidney releases significant amount of glucose in post-absorptive state[30]. The kidney was once thought to contribute mainly to whole-body glucose production only during acidosis or prolonged starvation[6,18,26]. The role and contribution of the glucose production by the kidney in other physiological and pathological conditions have emerged[18,31]. The kidney accounts for 10% systemic gluconeogenesis in the absorptive phase; the rate rises to as much as 25% in the post-

absorptive phase[32]. Moreover, in the case of prolonged fasting, the kidney prevents and reverses hypoglycemia by a counter-regulatory process of increased gluconeogenesis and inhibition of glucose uptake[33]. Besides such adaptive changes, impaired renal insulin signaling/sensitivity affects renal gluconeogenesis[15]. Improving renal insulin sensitivity may reduce systemic glucose levels *via* gluconeogenesis inhibition [34]. In the postprandial state, the renal glucose release accounts for approximately 50% of the endogenous glucose release for several hours. These observations suggested that increased renal glucose release may play an important role in facilitating efficient liver glycogen repletion by permitting substantial suppression of hepatic glucose release. Hormones (notably insulin and catecholamines), substrates, enzymes, and glucose transporters are some of the other factors which affect glucose production by the kidney[31,35-39].

The kidney differentially regulates glucose levels in the medulla and the cortex, with glucose utilization in the renal medulla and glucose production in the kidney cortex[19]. The separation of these processes is based on the differences in the distribution of various enzymes. The nephrons present in the renal medulla have glucose-phosphorylating and glycolytic enzymes; thus, they are involved in the phosphorylation and accumulation of glycogen. However, these cells lack gluconeogenic enzymes, and therefore, cannot synthesize or release free glucose into the circulation. On the other hand, renal cortex cells, more precisely the proximal tubule cells, possess gluconeogenic enzymes, and can produce and release glucose[26,40]. Therefore, the net equilibrium of glucose in the kidney is represented by the difference between renal glucose release by the cortex and renal glucose uptake by the medulla (Figure 1).

LOCALIZATION AND REGULATION OF KEY GLUCONEOGENIC ENZYMES IN THE KIDNEYS

PEPCK, FBPase, G6Pase, and pyruvate carboxylase catalyze the irreversible steps in gluconeogenesis. All these key enzymes are exclusively expressed in the S1–S3 segments of the proximal tubule[41-43]. PEPCK enzymes exist in two isoforms: cytosolic and mitochondrial. These enzymes are encoded by the two nuclear genes. According to human data, 60% of PEPCK is confined to mitochondria, while 40% to cytosol[44]. The cytoplasmic form is regulated at the transcriptional level by nutritional and hormonal stimuli, whereas the expression of mitochondrial form remains constitutive[45] (Figure 2). These three key enzymes are rate-limiting and, under metabolic alterations, PEPCK has been most extensively reported to be regulated. For example, in acidotic conditions, the expression and the activity of renal PEPCK have been found to be upregulated, while G6Pase and FBPase were marginally regulated[15,23,46]. Similarly, under insulin resistance conditions, PEPCK expression increased significantly compared to the levels of FBPase and G6Pase[12,15]. Further, the PEPCK/PCK1 activity in the kidney and the liver of diabetic patients correlates with the levels of PCK1 mRNA, with PEPCK and G6P being regulated at the post-transcriptional level, while FBP being regulated at the pre-or the post-translational level[8,47,48]. PEPCK and G6Pase have been shown to be transcriptionally regulated by a complex network of transcription factors and cofactors, including CREB, HNF-4α, and FOXO1[49].

RENAL GLUCONEOGENESIS IN THE POST ABSORPTIVE AND POSTPRANDIAL STATE

As discussed in the above sections, kidneys contribute significantly towards the total endogenous glucose production in normal physiological conditions, including fasting and postprandial states[26,50]. After an overnight fast, 75% of glucose entering the circulation is released by the liver, and the remaining 25% is released by the kidney[19,32,51].After a prolonged fast of 48 h, liver glycogen stores are depleted, and renal gluconeogenesis becomes the major source of glucose that is released into the circulation[51,52].Thus, as the duration of fasting increases, the overall proportion of glucose released *via* renal gluconeogenesis increases[53]. A few studies based on glucose release and glucose uptake by metabolic tissues suggest that the postprandial phase is also important in regulating glucose homeostasis. For example, a 61% decrease in overall glucose release *via* hepatic glycogenolysis was reported previously

Figure 1 Schematic overview of renal gluconeogenesis and glycolysis pathway and enzyme localization. The key enzymes of gluconeogenesis (1) pyruvate carboxylase; (2) phosphoenolpyruvate carboxykinase; (3) fructose-1,6-biphosphatase; and (4) glucose 6-phosphatase are predominantly localized in the renal cortical cells whereas, the glycolytic key enzymes (1) hexokinase; (2) phosphofructokinase; and (3) pyruvate kinase are found in the renal medulla.

in a human study, virtually ceasing in 4 to 6 h[54]. This finding was attributed to the need for replenishing the liver glycogen stores and to limit postprandial hyperglycemia. Moreover, unlike the liver, renal gluconeogenesis increases by approximately two-folds and accounts for 60% of endogenous glucose release in the postprandial phase[54]. The tight hormonal regulation helps maintain a homeostasis between the renal glucose release and uptake. Postprandial plasma glucose levels are majorly regulated by insulin and glucagon levels[32]. In another study, a four-fold increase in insulin and up to 50% decrease in plasma glucagon levels were observed after glucose ingestion in humans[55,56]. This process of mutual-regulation of glucose homeostasis is termed as hepatorenal glucose reciprocity. The term can be defined as a physiological or pathological decrease in glucose release by either one of the tissues-kidney or liver- with a linear increase in glucose release by the other[5]. Such situation is encountered during anhepatic phase post-liver transplantation, prolonged fasting, acidosis, meal ingestion, and insulin overdoses in diabetes mellitus[5,57,58].

INSULIN-MEDIATED REGULATION OF RENAL GLUCONEOGENESIS

Insulin has been demonstrated to attenuate enhanced renal gluconeogenesis in rodent models of type 1 diabetes[59,60-66]. Insulin is a known suppressor of gluconeogenesis in both, liver and kidney; however, kidneys are more sensitive to the suppressive effects of insulin[67]. Using the combined isotopic and net balance approach, insulin was shown to suppress renal glucose release and stimulated renal glucose uptake by 75% in conscious dogs[28]. A human study also showed that administration of insulin inhibitor increased renal glucose production in type 1 diabetic patients[19]. At molecular levels, insulin has been demonstrated to reduce the mRNA expressions of PCK1 and G6P[59]. This inhibitory effect is mediated through phosphorylation of FOXO1 *via* the IRS/Pi3k/Akt/FOXO1 pathway[59,68].Insulin inhibits the availability of gluconeogenic substrates or redirect the substrates to the oxidative pathways

Figure 2 Gluconeogenesis Pathway and cellular compartmentalization of the gluconeogenic enzymes. Pyruvate from lactate enters mitochondria by mitochondrial pyruvate transporter. Pyruvate provided by alanine transamination or lactate dehydrogenation is converted to oxaloacetate (OAA) by mitochondrial pyruvate carboxylase. OAA is either reduced to malate and exported out in the cytoplasm by malate ketoglutarate transporter or directly converted to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PCK) 2 (mitochondrial isoform) and exported out in the cytoplasm. In the cytoplasm, malate is first oxidized to OAA and then converted to PEP by PCK1 (cytoplasmic isoform). Fructose-1,6-bisphosphate (FBP) is then converted to fructose-6-phosphate by cytoplasmic FBP1. Glucose-6-phosphatase in the cytoplasm ultimately dephosphorylates glucose-6-phosphate to release glucose. G6Pase: Glucose-6-phosphatase; LDH: Lactate dehydrogenase; MPC: Mitochondrial pyruvate carrier; ALT: Alanine aminotransferase; FBP: Fructose-1,6-bisphosphate; OAA: Oxaloacetate; PCK: Phosphoenolpyruvate carboxykinase; PEP: Phosphoenolpyruvate; mMDH: Malate dehydrogenase; cAMP: Cyclic adenosine monophosphate.

[6,26,28]. Moreover, it indirectly affects glucose release *via* reduction of free fatty acid uptake[6,69,70]. A few reports have documented an inhibitory effect of insulin on renal gluconeogenesis through the substrates glycerol and glutamine in the post-absorptive state in humans[6,28]. However, regulation of renal gluconeogenesis by insulin, glucagon, and epinephrine is not widely studied in humans[6,71,72].

In the liver, the role of insulin or insulin receptor (IR) signaling in transcriptional regulation of gluconeogenic genes, that is, PCK1 and G6PC, is well known[73,74]. However, only a handful of studies have investigated the role of insulin *via* IR signaling in renal gluconeogenesis regulation. DeFronzo*et al*[75] reported the inhibitory effect of insulin on renal gluconeogenesis. Previously, we demonstrated high blood glucose and renal gluconeogenic-enzyme upregulation in mice with targeted deletion of IRs from the proximal tubule[13,59]. These IR knock-out (IRKO) mice exhibited normal insulin sensitivity, throughout their bodies. Additionally, increased activity and elevated mRNA expression of G6Pase observed in the IRKO mice indicates the role of the IR in regulating renal gluconeogenesis. In another study, reduced IR expression with a concomitant increase in PEPCK levels were reported in the kidney cortex of mice with high-fat-induced insulin resistance[76].In addition, *in vitro* studies in primary human proximal tubule (PT)cells also revealed insulin's inhibitory action on cAMP/DEXA-induced gluconeogenesis, while silencing of the IR attenuated this inhibitory effect[65] (Figure 3). Further down the signaling mechanism, Nakamura *et al*[77] demonstrated that, unlike the liver, insulin-induced inhibition of proximal tubule gluconeogenesis inhibition might be mediated *via* the IRS1/ Akt2/mTORC1/2 pathway. In another study, IRS2 (IRS2–/–) knockdown has been shown to result in elevated blood glucose levels in mice[78].However, the post-receptor signaling mechanism for insulin-induced inhibition of renal gluconeogenesis is not yet clear. Nevertheless, these studies indicate the significance of IR signaling in renal gluconeogenesis and suggest that defect in IR signaling to the kidneys may contribute to hyperglycemia in insulin resistance state[9-13,79].

Figure 3 siRNA mediated knockdown of insulin receptor in the human proximal tubule cells increased glucose production *via* gluconeogenesis stimulation.A: Western blot showing reduced insulin receptor insulin receptor (IR) expression in IR-siRNA treated human proximal tubule (hPT) cells relative to scrambled; B:cAMP/Dexa induced gluconeogenesis/glucose production in the hPT cell culture media; and C: Relative phosphoenolpyruvate carboxykinase mRNA transcript levels in scrambled and IR-siRNA treated hPT cells with or without insulin treatment. "Citation: Pandey G, Shankar K, Makhija E, Gaikwad A, Ecelbarger C, Mandhani A, Srivastava A, Tiwari S. Reduced Insulin Receptor Expression Enhances Proximal Tubule Gluconeogenesis. *J Cell Biochem* 2017; 118: 276-285 [PMID: 27322100 DOI: 10.1002/jcb.25632] Copyright © The Author(s) 2017. Published by John/Wiley & Sons, Inc[65]"

RENAL GLUCONEOGENESIS IN CASES OF INSULIN RESISTANCE AND DIABETES

Insulin resistance refers to inefficient sensitivity of primary metabolic tissues towards insulin and is characterized by a reduced insulin action despite hyperinsulinemia [80-82]. Like the other metabolic tissues, kidneys also lose their insulin sensitivity during insulin resistance[14,61,83].The mechanism of insulin resistance is different among different organs and even cells of the same organ. For example, in case of insulin resistance, IRS2 signaling is impaired in liver too. However, in the renal proximal tubules, insulin signaling *via* IRS1 is impaired; however, the signaling *via* IRS2 is preserved[84-87].

Insulin resistance has frequently been associated with renal abnormalities, such as impaired glucose metabolism[12,79,88]. These studies suggest that impairment of the expression or post-receptor signaling of the IR can enhance renal gluconeogenesis in the diabetic patients. A wide distribution of IR throughout the nephron segments and their reduced expression in renal epithelial cells in insulin resistance models have been reported[14].We and others have demonstrated reduced expression of IR and its phosphorylated form in the kidney cortex of diabetic rodents and humans [14,61,65,89]. In a previous study, newly diagnosed cases of type-2 diabetes were reported to exhibit impaired insulin-induced suppression of gluconeogenesis [9,11,79]. Our recent study also suggested impairment in meal-induced inhibition of renal PEPCK in individuals with reduced insulin sensitivity[15].Thus, insulin resistance might be responsible for high levels of gluconeogenic enzymes found in

renal biopsies from T2D human and rodent models[61,65,90].

Nevertheless, impaired IR signaling to the kidneys also affects kidneys' vital functions, including the endogenous glucose production by the kidneys[13,91-93]. We previously reported altered systemic glucose metabolism in IRKO mice, which further strengthens this proposition[13].Thus, similar to the liver, insulin resistance could impair renal gluconeogenesis in diabetes patients[14,61]. Previous studies on diabetic animal models have reported increased renal gluconeogenic enzyme activity and glucose release[48,94-98]. In 1999, Meyer reported significantly higher systemic glucose levels in diabetic patients compared to normal subjects, of which 40% of glucose content was contributed by renal glucose release[16]. Another *in vitro* study conducted by Eid *et al*[12], for the very first time, reported increased gluconeogenesis in the proximal tubules of obese Zucker rats. Another *in vivo* study reported an intrinsic increase in renal gluconeogenesis and increased PEPCK mRNA levels in type 2 diabetic model[12,61,83,99].The other key enzymes, FBPase and G6Pase, were, however, marginally regulated[12] (Figure 4). Moreover, recent rodent model studies conducted by us and others also indicated the significant role of renal gluconeogenesis in fasting hyperglycemia[13,15,59,65]. Furthermore, increased renal gluconeogenesis contributed to increased level of fasting glucose in T2DM patients and raised postprandial glucose. Furthermore, many human studies also reported an increase in the release of glucose by the kidney in the fasting state in T2DM patients[100-104], which might be attributed to gluconeogenesis[105].Additionally, abnormal postprandial glucose metabolism has also been reported in T2DM patients[16]. In this study, dual-isotope and net balance measurement across kidney, liver, and skeletal muscles revealed an impaired suppression of gluconeogenesis by kidney and liver, leading to increased levels of postprandial glucose. The other possible reasons for this postprandial increase in glucose levels in type 2 diabetic condition include persistently increased glucose levels in the post-absorptive state[106],high levels of free fatty acids, and increased substrate availability[54,61,105,107,108].

CLINICAL MANAGEMENT

Insulin resistance is a known risk factor for developing pre-diabetes, and eventually, type-2 diabetes. Insulin resistance at the kidney level could further contribute to hyperglycemia by enhancing renal gluconeogenesis. Thus, improving insulin sensitivity *via* lifestyle modifications, such as dieting and physical activity, could be a preventive strategy for pre-diabetes and improving glycemic levels in diabetes patients. Two classes of drugs, biguanides and thiazolidinediones, are available commercially for improving insulin sensitivity. In clinical practice, both these agents are in common use for glucose-lowering in patients with type-2 diabetes[26,109,110]. By enhancing renal insulin sensitivity, these agents exhibit great potential in regulation of renal function in T2DM patients[111,112]. Apart from the known insulin sensitizers, SGLT2 inhibitors are emerging as another promising anti-hyperglycemic agent. They induce glucosuria by inhibiting glucose reabsorption in the renal proximal tubules[113]. Inhibition of renal glucose reabsorption and induction of glucosuria by these agents are considered to be effective and safe in patients with T2DM. Moreover, their insulin-independent action lowers hypoglycemia risk commonly associated with other anti-diabetic drugs[26].

Interestingly, SGLT2 inhibitors have been postulated to act by modulating insulin sensitivity and/or renoprotective actions in T2DM patients[114]. Dapagliflozin, an SGLT2 inhibitor, has been shown to improve renal function and renal insulin signaling in an animal model of diet-induced obesity[115]. Dapagliflozin, either as monotherapy or add-on therapy to insulin or metformin, was found to reduce glucose and HbA1c levels in T2DM in clinical trials[116]. Also, dapagliflozin or empagliflozin, along with insulin therapy, imparts clinical benefits in patients with type-1 diabetes[117,118]. However, more studies are warranted to confirm their therapeutic potential as an adjunct therapy.

CONCLUSION

Renal gluconeogenesis plays a key role in normal physiology, where its impairment contributes adversely with pathological implications. Overall, this review suggested enhancement or insulin-mediated impairment of renal gluconeogenesis in cases of insulin resistance. Such impairment may further contribute to hyperglycemia in type-2

Figure 4 mRNA and protein levels of glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and fructose-1,6-bisphosphatase in diabetic rats and their non-diabetic controls. "Citation: Eid A, Bodin S, Ferrier B, Delage H, Boghossian M, Martin M, Baverel G, Conjard A. Intrinsic gluconeogenesis is enhanced in renal proximal tubules of Zucker diabetic fatty rats. *J Am SocNephrol* 2006; 17: 398-405 [PMID: 16396963 DOI: 10.1681/asn.2005070742] Copyright © The Author(s) 2006. Published by the American Society of Nephrology Inc[12]"

diabetes. However, more research is warranted in this area to further elucidate the associated mechanism.

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Fear of hypoglycemia, a game changer during physical activity in type 1 diabetes mellitus patients

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Abstract

Hypoglycemia limits optimal glycemic management of patients with type 1 diabetes mellitus (T1DM). Fear of hypoglycemia (FoH) is a significant psychosocial consequence that negatively impacts the willingness of T1DM patients to engage in and profit from the health benefits of regular physical activity (*e.g.*, cardiometabolic health, improved body composition, cardiovascular fitness, quality of life). Technological advances, improved insulin regimens, and a better understanding of the physiology of various types of exercise could help ameliorate FoH. This narrative review summarizes the available literature on FoH in children and adults and tools to avoid it.

Key Words: Hypoglycemia; Fear of hypoglycemia; Type 1 diabetes mellitus; Physical activity; Modern technology; Exercise management

Core Tip: Fear of hypoglycemia (FoH) is a major barrier to achieving recommended

childhood and adult levels of physical activity (PA) in type 1 diabetes mellitus patients. The main factor associated with FoH is the frequency of hypoglycemia. The occurrence of hypoglycemia can be mitigated by well-defined recommendations on performing PA and maintaining euglycemia, implementing modern technologies and improved insulin regimens, educational programs, and social media information.

INTRODUCTION

Hypoglycemia, a barrier to glycemic management in type 1 diabetes mellitus

Intensified insulin regimens aim to achieve a near-normal glucose level, which is the primary goal of type 1 diabetes mellitus (T1DM) management because it is associated with a decreased risk of long-term chronic micro- and macrovascular complications. On the other hand, intensive treatment increases the risk of acute side effects, such as hypoglycemia, which in itself correlates with increased morbidity and even mortality[1,2]. Hypoglycemia is often the limiting factor in the glycemic management of T1DM patients[3], and it can compromise patients' willingness to maintain a healthy lifestyle, primarily adversely affecting involvement in regular physical activity (PA)[4]. Reducing the risk of hypoglycemia and, at the same time, maintaining or improving glycemic control is imperative. Patient education, empowerment, modern technology, improved insulin regimens, and healthcare workers' support are deemed necessary.

BENEFITS OF PA FOR PATIENTS WITH T1DM

Regular PA beginning in early childhood is important for both physical and psychological development. For individuals with T1DM, PA offers many health benefits, including improved glycemic control and blood lipid profiles, better cardiovascular function, and psychological wellbeing[5,6]. Unfortunately, most people with T1DM do not regularly engage in adequate PA. As a direct or indirect consequence, as many as 60% of patients have hyperlipidemia, 40% have hypertension, and 60% are obese or overweight, thus having an increased cardiovascular risk[7]. On the other hand, the results of an extensive cross-sectional study suggest that regular PA that includes exercising ≥ 2 times weekly can improve cardiometabolic parameters and reduce microvascular complications (*e.g.*, nephropathy-microalbuminuria or retinopathy) of diabetes[8]. Exercise intervention studies have generally failed to show clinically meaningful improvements in hemoglobin A1c (HbA1c) with PA in T1DM[9]. That might be attributable to differences in study design and exercise dosage as well as adjustments of insulin dosing and/or carbohydrate intake while exercising intended to minimize exercise-induced hypoglycemia and ensure safe blood glucose levels[10]. The study by Dube *et al*[11] found that people with T1DM who were engaged in moderate or intense PA reported increased HbA1c, carbohydrate consumption, and weight gain, which was explained by their tendency to avoid hypoglycemia. Therefore, hypoglycemia and fear of hypoglycemia (FoH), in addition to lack of knowledge of exercise management in T1DM, are significant obstacles in reaching recommended PA targets.

IS FOH STILL THE MAIN OBSTACLE TO ACHIEVING PA TARGETS?

FoH encompasses an anxiety disorder in patients and their families that is caused by hypoglycemia and the associated behavioral changes affecting glycemic management, such as avoiding exercise, maintaining high blood glucose levels, and administering

low insulin doses[12]. FoH can have a severe impact not only on regular exercise but also on all aspects of life quality. Anxiety is the most common consequence of hypoglycemia-induced distress leading to two potential scenarios. One could be adaptive, instigating productive behavior regarding glucose management[13,14]. However, that could also be disruptive, triggering behavioral changes leading to poor glycemic control and impaired quality of life[15]. So far, it has been shown that, FoH frequently occurs in parents who report severe hypoglycemia in their children[16,17]. Several studies have confirmed a positive correlation between FoH and HbA1c in young children and adolescents and that high parent and adolescent scores in an FoH survey were associated with increased HbA1c levels[18-20]. Other factors affecting parental FoH were nonmodifiable sociodemographics (*e.g.*, age, education, nationality) and modifiable psychological factors (*e.g.*, mindful parenting)[21]. A study assessing FoH in adult patients identified the frequency of severe hypoglycemia as the most relevant factor associated with FoH[22]. In addition, several predictive factors such as female gender, hypoglycemia unawareness, and glucose variability seem to be associated with hypoglycemia[23]. Moreover, FoH was recently shown not to be time-dependent in most cases, but to be conditioned by change in hypoglycemia frequency, which emphasizes the significance of mitigating hypoglycemic events[24]. Results of the Diabetes MILES study in the Netherlands have shown that FoH in adults with T1DM was associated with a history of hypoglycemia and depressive symptoms[25]. Interestingly, although FoH is a major obstacle to performing regular PA, there are few data on FoH and exercise. However, existing studies identified FoH as a major culprit for inadequate exercise[26-28]. Surprisingly, in the SEARCH for Diabetes in Youth Case-Control Study, 82% of participants with T1DM achieved the recommended PA goals compared to healthy peers[29], in contrast to the 33% of young adults with T2DM who achieved the recommended PA targets[7]. Recently published data showed that increased levels of vigorous PA (VPA) were associated with increased FoH scores in SEARCH patients with T1DM. In addition, there was a decrease in VPA with age and an increase in moderate PA[30]. Therefore, we can assume that FoH does not affect PA or that VPA is considered a better option, given recent recommendations that include the benefits of high intensity interval training (HIIT) to avoid hypoglycemia[31]. In the same study, HbA1c positively correlated with pediatric FoH, resulting in poor glycemic control. On the other hand, lower HbA1c correlated with higher VPA, emphasizing a well-known positive effect of PA on glucose management[32].

PA: RECOMMENDATIONS FOR PATIENTS WITH T1DM

Although the recommendations on PA for people with T1DM and those with type 2 diabetes mellitus do not differ, some specifics need to be considered. In general, 150 min of accumulated aerobic PA is recommended weekly, with no more than 2 consecutive days without PA plus resistance training two to three times weekly, but not on consecutive days[33]. For children and adolescents, the recommendation is to be involved in PA at least 60 min daily[34,35].

What makes the difference in managing PA in the case of T1DM are the specific patient goals for exercise (*e.g.*, metabolic control, prevention of complications, fitness, weight loss, or competition and performance), which should be considered before decisions on diabetes management are made. For example, strategies behind exercising for weight loss should focus on reducing insulin doses during and after exercise instead of consuming carbohydrates. On the other hand, if sports results and exercise performance are the primary goals, nutritional guidance specific to the sporting activity is most important, while modifications in insulin dosing are secondary to match the new/additional nutritional requirements. Regardless of the set goals, blood glucose monitoring before, during, and after exercise is essential to avoid hypo and hyperglycemia[36-38]. Despite the perceived benefits, many young people with T1DM do not meet the proposed PA recommendations[39], and it is still unclear what would be the most effective type of exercise for improvement of metabolic control and cardiovascular health in people with T1DM[40,41].

From a practical standpoint, to minimize the risk of hypoglycemia, glycemic levels during, but also before and after exercise should be individually tailored. For the majority of patients with T1DM, the starting glucose range should be somewhere between 7-10 mmol/L when initiating aerobic exercise lasting up to an hour. When engaging in HIIT and anaerobic exercise, training can be initiated at the lower starting glucose concentration range, somewhere between 5-7 mmol/L, as during this kind of

exercise, glucose levels either remain stable or even tend to rise[31]. Although it is still unclear whether there is an optimal glycemic range during exercise, available data suggest that concentrations between 6-8 mmol/L should be preferred[42]. Strategies to prevent hypoglycemia rely on previous hypoglycemic episodes, which should be taken into account when planning subsequent PA[43]. An episode of severe hypoglycemia within the previous 24 h presents a contraindication to exercise[44]. As insulin sensitivity increases after exercise (and can remain increased for up to 48 h[45]), strategies to minimize exercise-related hypoglycemia, especially nocturnal occurrence, should be employed, including the avoidance of the exercise during the late afternoon[46-48].

PREVENTION OF HYPOGLYCEMIA-A ROLE FOR IMPROVED INSULIN REGIMENS AND MODERN TECHNOLOGY

Regular PA is usually recommended as an integral part of diabetes management in children and adults with T1DM[33,35]. However, it is frequently accompanied by unwanted blood glucose changes and requiring a range of potential preventive measures. Even if implemented, precautions are not always sufficient to avoid excessive glucose excursions. Moreover, handling different forms of PA can be particularly challenging for both individuals with T1DM and healthcare providers, whereby the decision-making process is often trial and error based[49]. In younger children, the tricky part about PA is that it is often unplanned and unpredictable.

Among the well-known barriers to regular PA, FoH and loss of glycemic control are shared by adults and youth with T1DM[26,27,31]. Consequently, the need for education on safe PA practices is widely recognized and acknowledged[30,31,50,51]. Recent advances in insulin formulations, delivery methods, continuous glucose monitoring (CGM) systems, applications (apps), and algorithms that integrate novel technologies are expected to improve glycemic control with less hypoglycemia and a better quality of life for people with T1DM.

Insulin therapy has been the cornerstone of diabetes management for almost a century, and despite the current availability of a wide array of insulin preparations, significant unmet needs remain[52]. With rapid-acting insulin analogs (*e.g.*, insulin aspart, lispro, glulisine), a reduction in bolus dose accompanying the meal before exercise is still required. On the other hand, with new long-acting basal insulin analogs (*e.g.*, degludec, glargine U300), dose adjustments for PA are impractical and cannot be performed without overall glycemic control disturbance[31]. A glucose-responsive insulin patch, an innovative and promising treatment option already successfully tested in diabetic animals, is awaiting clinical trials in humans.

In recent years there has been a great expansion of diabetes-specific technology, including CGM systems, insulin pumps, and automated insulin delivery systems. Glycemic management during exercise has been made easier with CGM technology. Most common CGM systems measure glucose in the interstitial fluid, providing real-time sensor glucose data (rtCGM) and triggering alerts for hypo and hyperglycemia. Intermittently scanned CGM systems (isCGM) measure interstitial glucose levels at the time of scanning and lack alarms. A lag time between blood and interstitial fluid glucose value exists and is particularly pronounced when blood glucose levels change rapidly, that occur during exercise. Furthermore, physiological changes during PA, such as alterations in blood flow rate, body temperature, and acidity, can theoretically disturb interstitial glucose-sensing accuracy[53]. General recommendations can be used as an initial guidance tool when using rtCGM/isCGM before exercise, during exercise, after exercise, and the nocturnal post-exercise phase[54]. However, for different groups of people with T1DM, different glycemic ranges around exercise may be required. Finally, all recommendations should be tailored individually, relying on the use of sensor glucose values accompanied by trend arrows while employing safe sensor glucose thresholds[54].

Insulin pumps offer better flexibility in insulin dose adjustments and management of exercise-associated glucose excursions than other insulin delivery methods[55-57]. Basal rate reduction mitigates the risk of hyperglycemia after moderate exercise more effectively than basal insulin suspension and appears to be associated with reduced risk of hypoglycemia both during and after PA[58]. However, the optimal timing and percentage of basal rate reduction have to be individually determined. Besides, for a more effective reduction of circulating insulin levels during PA, the remaining insulin from the bolus applied within the previous 2 h can be considered[59]. For intense exercise, the best option is to temporarily stop the pump, which coincides quite nicely

with the preference of patients who wish to remove their pump during activity. To prevent early exercise-related hypoglycemia after the meal, the premeal bolus dose reduction might be more effective than reducing the basal rate[58].

Sensor-augmented pump therapy combines insulin pumps, CGM systems, and therapy management software to automatically suspend insulin delivery for up to two hours when CGM detects a glucose level that has reached a prespecified threshold or to suspend insulin in anticipation of hypoglycemic events. In recent years, those devices have been connected to create a more automated glucose monitoring and insulin dosing combo marketed as a closed-loop system that automatically adjusts basal insulin delivery based on CGM readings[60]. Several studies have shown a reduction in hypoglycemia incidence and severity in patients using closed-loop systems in pediatric and adult patients[61,62]. However, although effective and highly promising, these systems warrant further research for an optimized use around exercise[63].

Digital health technology is developing rapidly, and numerous health-related apps installed on smartphones or other wireless devices are already available to support people with diabetes in lifestyle interventions or insulin adjustments in response to glucose monitoring data[64]. Several apps are primarily intended to support PA in people with T1DM, as they allow users to track activity, count calories, and set goals for exercise and weight management. However, the available evidence on the safety and efficacy of such stand-alone diabetes apps is still limited. Several issues, including inadequate or insufficient information on app accuracy, clinical validity, data security, and lack of user training, need to be resolved to ensure the full potential of such diabetes apps[65].

CONCLUSION

FoH-are we ready to discard it?

General guidelines for minimizing exercise-related glucose excursions exist, but their implementation is often burdensome for people with T1DM. As numerous factors influence glycemia during exercise, such as glucose level at the start of PA, the type, intensity, and duration of exercise as well as its' timing concerning meals, all the preventive actions require major individualization and are not always successful in avoiding hypoglycemia. Optimizing glycemic control around exercise is still demanding. Despite many advances in insulin formulations, delivery methods, and CGM systems, a thorough education is still essential. As technology continues to progress, people with T1DM are expected to achieve better glycemic control around exercise with less hypoglycemia and a lighter mental burden. However, many questions regarding availability, affordability, and adherence to expanding diabetes technology remain. Although FoH remains one of the main obstacles in achieving recommended PA levels, according to the latest research in young populations, it could even encourage more vigorous exercise patterns. Possibly, the development of new technologies, educational programs and social media information on the importance of PA, updated recommendations regarding the type and time of exercise, and necessary adjustments allowing euglycemia during and after the exercise have had a significant impact on alleviating FoH. Hopefully, future research embracing all of these FoH related aspects will enable us to minimize or disregard the problem of FoH. Finally, our understanding of exercise physiology and the different effects aerobic, anaerobic, resistance training and HIIT have on glycemic levels can minimize the risk of hypoglycemia and improve T1DM management, especially in the setting of new technology and improved insulin on board (Figure 1).

Figure 1 Potential mechanisms that can be used to minimize the frequency and fear of hypoglycemia to help type 1 diabetes mellitus patients reach the proposed physical activity levels and improve physical and psychological health. FoH: Fear of hypoglycemia; HYPO: Hypoglycemia; PA: Physical activity; QoL: Quality of life.

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Chronic care model in the diabetes pay-for-performance program in Taiwan: Benefits, challenges and future directions

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Abstract

In this review, we discuss the chronic care model (CCM) in relation to the diabetes pay-for-performance (P4P) program in Taiwan. We first introduce the 6 components of the CCM and provide a detailed description of each of the activities in the P4P program implemented in Taiwan, mapping them onto the 6 components of the CCM. For each CCM component, the following three topics are described: the definition of the CCM component, the general activities implemented related to this component, and practical and empirical practices based on hospital or local government cases. We then conclude by describing the possible successful features of this P4P program and its challenges and future directions. We conclude that the successful characteristics of this P4P program in Taiwan include its focus on extrinsic and intrinsic incentives (*i.e.*, shared care network), physician-led P4P and the implementation of activities based on the CCM components. However, due to the low rate of P4P program coverage, approximately 50% of patients with diabetes cannot enjoy the benefits of CCM-related activities or receive necessary examinations. In addition, most of these CCM-related activities are not allotted an adequate amount of incentives, and these activities are mainly implemented in hospitals, which compared with primary care providers, are unable to execute these activities flexibly. All of these issues, as well as insufficient implementation of the e-CCM model, could hinder the advanced improvement of diabetes care in Taiwan.

Key Words: Chronic care model; Diabetes; Pay-for-performance; Shared care; Diabetes care

Core Tip: Most studies have shown that pay-for-performance (P4P) can reduce diabetes-related complications. The successful characteristics of this P4P program in Taiwan include its focus on extrinsic and intrinsic incentives (*i.e.*, shared care network), physician-led P4P and the implementation of activities based on the chronic care model components. However, the P4P coverage rate should be steadily improved, and Taiwanese government should invest more in primary care to help these facilities participate in the P4P program and have the capacity to implement chronic care model-related activities.

INTRODUCTION

There are two kinds of pay-for-performance (P4P) designs[1]. The first type of design is called indicator-based P4P, which is designed to provide extra incentives and establish a fee for service or capitation to meet objectives; examples are the P4P systems adopted in the United Kingdom[2], the United States [not including patient-centered medical homes (PCMH)][3], France[4], South Korea[5], *etc*. The second type is called participatory P4P, which is especially for diseases such as diabetes and has been employed in the PCMH model in the United States[6], Australia[7], Ontario of Canada[8], Tuscany in Italy[9], and Taiwan; this design is focused on patient engagement and simply rewards participation in care-improvement activities without necessarily linking bonuses to the attainment of objectives based on specific measures. Both of these types of P4P designs have their own distinct advantages. For example, the former design can steer the provider toward the predefined goals, and the latter design places fewer limitations on professional autonomy and fosters cooperation between providers and other medical staff (*e.g.*, health educators)[1,10]. In general, the effect of P4P designs on outcomes is still arguable, and most studies have been conducted in the United States and the United Kingdom[11]. Participatory P4P research outside United States and United Kingdom is still in its infancy, and further research is needed[1].

Participatory P4P achieves better outcomes

In participatory P4P, some of these countries have achieved better outcomes for diabetes; for instance, in 2010, the Tuscany region implemented this kind of P4P design to improve general practitioner (GP) management of chronic diseases[9]. In Taiwan, more than 10 studies have shown that this kind of P4P design can reduce negative outcomes. For example, most studies have shown that P4P can reduce diabetes-related complications[12-14], emergency department visits or hospital admissions[15,16], the incidence of cancer[17], tuberculosis[18], and mortality[12,19-21]; improve HbA1c control[22]; and achieve cost-effectiveness[23-25]. The achievements of diabetes P4P designs have received attention from international scholars and the media, such as the Economist[26,27]. The diabetes P4P program in Taiwan not only aligns the extra incentive provided to physicians with good compliance with diabetes but also, most importantly, integrates chronic care model (CCM)-oriented activities, which are carried out by physicians as the leaders of professional teams.

The importance of this review

In Taiwan, most studies related to the diabetes P4P system have tended to focus on the effectiveness of the integrative model (*i.e.*, have mainly focused on outcomes)[28]. However, a detailed understanding of the P4P system in Taiwan is more important because it may provide insights into how to build a P4P system featuring incentives for care compliance and care activities based on the CCM. In addition, if the beneficial

activities associated with all CCM components are clearly mapped from a nationwide perspective, the subsequent implementation of these activities could be more informed[29]. Moreover, recent scoping research has indicated that empirical studies on the successful application of the CCM have mainly focused on two dimensions, namely, patient self-management and provider service delivery[30], and have addressed other components less. Hence, in this review, we aim to discuss the CCM in the diabetes P4P program in Taiwan. We first introduce the 6 components of the CCM and provide a detailed description of each of the activities in the P4P program implemented in Taiwan, mapping them onto the 6 components of the CCM. For each CCM component, the following three topics are described: the definition of this specific CCM component, the general activities implemented related to this component, and practical and empirical practices based on hospital or local government cases. We then conclude by describing the possible successful features of this P4P program and its challenges and future directions.

METHODLOGY

The 6 components of the CCM

Many of the initial disease management strategies for improving quality of care were cooperative and were referenced in Wagner's CCM proposed in mid-1990[31]. Many activities implemented by disease management programs for improving chronic care have been based on the different levels and components of the CCM, which often includes four levels for classifying different activities: (1) the system; (2) the physician/facility; (3) the patient; and (4) the community[32]. Furthermore, the CCM has 6 interrelated components: (1) a health care system (at the system level); (2) a coordinated care/delivery system design (at the hospital/physician level); (3) a decision support system (DSS) (at the hospital/physician level) to support physician guideline adherence; (4) patient self-management (at the patient level); (5) community resources (at the community level); and (6) a clinical information system (CIS) (at the hospital/physician level)[32,33].

Mapping the activities in the diabetes P4P program in Taiwan to the CCM components

We searched PubMed using the following keywords: "share care/shared care/case management/care system/comprehensive care and Taiwan and diabetes". Articles found in the different searches were used as the materials for this review. For the mapping of activities to the 6 components of CCM, at least 9 articles can be referenced, and we described empirical practices based on these articles (see below)[28,34-41].

CCM component 1 — the health system in Taiwan (system level): The health system focuses on creating a culture, organization and mechanisms that foster productive interactions with consumers and promote safe, high-quality care, which includes incentives based on quality of care, visible support for improvements provided by senior leadership, and the development of agreements that facilitate care coordination within and across organizations[31,33,42,43]. We discuss incentives based on quality of care of CCM component 1 below in detail (the leadership issue will be mentioned in the Discussion section).

Regarding incentives based on the quality of care, the P4P program has undergone a two-stage evolution, with the initial establishment of disease management activities the shared care network (SCN) followed by the integration of reward systems for examinations and these activities. The pilot SCN proposed in Taiwan, called the Lan-Yang Diabetes Shared Care System (LYDSCS), which was first experimentally implemented in I-Lan County in 1996, was collaboratively executed by governmental authorities (the central Bureau of Health Promotion and local government) and hospitals[36]. After the successful implementation by I-Lan County, the National Health Insurance Administration (NHIA) integrated external incentives with this SCN as the first generation of the diabetes P4P program in 2001, which not only enforces the execution of suggested activities from the SCN but also highlights the adherence to guidelines for physicians to conduct the necessary examinations[35,36,41]. Based on the regulations for the incentive structure described in the 4th to 9th proposals for the diabetes P4P programs from 2006 to 2012, a team can receive a one-time sign-on payment of US $13 per patient enrolled in the P4P program at the hospital. In addition to the regular fee-for-service charge from the annual global budget, a team earns US $108 (not including the enrollment fees) for each patient who completes the cycle of

care in a year and whom the team sees at least four times per year. The incentives (US $108) include three follow-up fees (total US $21), a one-time yearly evaluation fee (US $27), and physician fees that are paid four times, once for every patient visit except for visits to stand-alone clinics (total US $60)[14].

The P4P proposal suggests once a patient was enrolled as a participant or had undergone a yearly assessment, the patient must be given 11 essential lab tests A1c, fasting plasma glucose, fasting lipid profile (total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein), serum creatinine, serum glutamic-pyruvic transaminase, urinalysis, microalbumin, and a dilated eye examination[14]. Regarding the three follow-up visits, only two necessary examinations need be done, the A1c and glucose. In August 2006, there was a physician-level outcome incentive for the two poor outcomes used, which were "Percentage of A1C ≥ 9.5 percent" and "Percentage of low-density lipoprotein ≥ 130 mg/dL"[44].

Regarding the development of agreements that facilitate care coordination within and across organizations, some local counties, such as Changhua, have expanded the role of public health nurses and dietitians in health centers to cooperate with private primary care physicians[22]. In addition, some small facilities cannot hire a diabetes team due to cost considerations; however, by sharing labor from large facilities with small facilities, adequate economies of scale can be attained[36].

CCM component 2 — patient self-management support/patient education in Taiwan (patient level): Patient self-management support includes the following activities: information provision, patient education (general, disease, and self-management), behavioral/motivational support, patient-centered care (*i.e.*, goal setting), and the provision of self-management tools[31,33,45].

The P4P proposal suggests the following evaluations, which fit with the principles above: (1) short- and long-term goals; (2) adherence to self-management training; (3) self-management of blood sugar results; (4) knowledge of diabetes; and (5) self-management skills. The P4P proposal also suggests the following educational steps that should be recorded into patients' charts: (1) describe the disease progression and treatment options; (2) make lifestyle changes and diagnose personal problems; (3) use a coping strategy to solve problems in daily life; and (4) implement patient self-management from these principles[14].

Special hospital cases to help patients perform self-management are as follows. (1) In the Case 1 hospital, education is conducted in a small group of 8-10 patients around a table using an interactive approach that is intended to resolve the problems associated with self-care, reinforce knowledge of diabetes, and encourage participants to share experiences. The content of discussion includes motivation to exercise and make dietary changes, the suggestion of at least 150 min/wk of exercise, and the selection of a type of exercise depending on the patient's lifestyle and preferences. Each patient is encouraged to monitor his or her own blood glucose[28]; And (2) In the Case 2 hospital, patients use a self-management tool called an insulin rotary disc, which is made by this hospital. Patients adjust their insulin dosage according to the suggestion displayed by the tool when the dial is aligned with the average of the past fasting glucose values[34].

CCM component 3 — a coordinated care/delivery system design/practice redesign in Taiwan (hospital/provider level): The coordinated care/delivery system design includes the following activities: team-based care provision (professionals play roles on a team), individualized care (case management), follow-up, adjustment based on health literacy and cultural background, and nurse/physician-led care[31,33,45].

The P4P proposal strictly requires that the care of diabetes patients be performed by a team consisting of trained professions[14]. The members of a team should include at least one physician of any specialty (endocrinology, family medicine or internal medicine), health educators (registered nurses), and dietitians, and they must register with the division of health of the local government. Communication between team members is facilitated through clinical training, periodic conference meetings, panel discussions, and the circulation of newsletters, and physicians within the P4P system use shared referral protocols and referral sheets[36,40]. In addition, the proposal also suggests that the delivery system be tailored to make psychosocial adjustments according to the patient's condition[14]. Through training in the relevant knowledge and skills above, in 2013, more than 4000 health professionals, including physicians, nurses, dieticians, and pharmacists, were qualified by the Taiwan Association of Diabetes Educators (TADE), which indicates that they completed the training to gain the relevant knowledge and skills, as well as the Taiwan Bureau of Health Promotion, Department of Health (this unit has now been promoted to the Ministry of Health and Welfare)[35].

Special hospital cases for redesigning delivery systems are as follows. (1) Regarding individualized care in the Case 1 hospital, taking nutrition therapy as an example, the dietitians in this hospital provide individualized nutrition plans that are prescribed based on recommendations and that are adjusted based on a patient's preferences; ideal body weight; and demographic, religious and socioeconomic factors[28]. Furthermore, the nurse case manager and dietitian in this hospital are sent to visit patients who express difficulty in identifying "high-sodium foods" in their diet[22]; And (2) In the Case 2 hospital, regarding adjustments based on health literacy, educational materials are translated into different languages; materials are also adapted to different literacy levels[34]. In addition, the hospital also focuses on patients' emotional well-being by supporting them as they adjust their psychological conditions and social relationships in daily life; the hospital receives patient feedback or elicits patients' feelings about insulin injection or glucose monitoring[34]. For remote patients, the same hospital even formed the Healthcare Diabetes e-Institute to enhance self-management through telecare for patients living in an underserved rural community[38].

CCM component 4 — the clinical information system in Taiwan (hospital/provider level): The CIS focuses on organizing data to facilitate efficient and effective care, including summarizing data to help track and plan care and facilitate performance monitoring and quality improvement efforts[31].

In Taiwan, systems that are used to monitor patient records or hospital performance, such as the CIS, are built at three levels. (1) At the national level, hospitals must report patient clinical outcomes *via* the virtual private network (VPN). The NHIA provides a website for every hospital to track and query their patients[41]; (2) At the local government level, some counties, such as Changhua County, have built the diabetes care management information system (DCMIS) to promote the use of clinical information in primary care. The DCMIS includes functions such as registration, reminders, descriptive statistics, and quality report production[39]; And (3) At the hospital level, hospital-made systems are usually richer than nationwide-level VPN systems. Hospitals can set up a diabetes registry that automatically captures their hospital information system records and monitors data for patient follow-up visits, such as patient demographics, telephone interview records, clinical chemistry values (outcomes), and health education records[34].

CCM component 5 — the DSS/expert system in Taiwan (hospital/provider level): The DSS focuses on promoting clinical care that is consistent with scientific evidence, which includes evidence-based guidelines for daily clinical practice and proven provider education methods[31].

In Taiwan, systems designed to provide performance feedback and/or reminders, such as the DSS, are built at two levels. (1) At the nationwide level, a public report card system is used in Taiwan[46] through which hospitals can receive feedback on diabetes quality, conduct benchmarking, and improve their performance[34,35,39]; And (2) At the hospital level, hospitals may make their own DSS, which can require the implementation of alert functions and reminders for guideline adherence[34,35]. All of these monitoring measures align with evidence-based guidelines.

CCM component 6 — community resources in Taiwan (community level): At the community level, the focus is on the mobilization of community resources to meet the needs of patients, which includes encouraging patients to participate in effective community programs and forming partnerships with community organizations to support and develop interventions that fill gaps in needed services[31].

Community resources focus on patient self-support groups, which have undergone 2 stages and have developed since the early era of LYDSCS. In the early period, the era of the LYDSCS brought about the planning of patient self-support groups[36]. With support from the Bureau of Health Promotion, TADE represents over 450 diabetic patient groups in different regions of Taiwan and aims to improve diabetes self-care and high risk awareness[22,36,41]. In the latter period, most patient peer groups were generated by the hospital itself. Hospitals invited patients' caregivers or peer groups to supervise patients, participate in the discussion groups, and provide information about compliance[28,37]. Based on these activities, patients' families or peer groups gradually became members of the medical team, and health professionals now consider them to be partners[40].

DISCUSSION

There are several policy implications for countries outside Taiwan, including the dual role of incentives as P4P features and physician-led quality improvement. However, these policy implications cannot be generalized throughout the world and are only referenced by countries with similar participatory P4P systems. In addition, there are also some disadvantages of diabetes P4P programs that need to be improved in the future (see the final paragraph).

Focus on extrinsic and intrinsic incentives (dual role)

Although reward systems are for both examinations and CCM-oriented activities in Taiwan, these kinds of incentives are mainly used for examinations because the low amount of incentives cannot sufficiently cover the cost of CCM-oriented activities. Extrinsic incentives (money) that target examinations alone without complex CCM-oriented activities are not sufficient to drive the improvement of patient outcomes [10,47] unless a large amount money is paid[11]. Usually, an extrinsic incentive is not sufficiently large enough to drive the execution of CCM-oriented activities, and compliance regarding the execution of these activities should also rely on the power of intrinsic incentives. Physicians have intrinsic motivations, such as professional autonomy/professionalism, inherent desires, and providing good-quality care, for performing these CCM-oriented activities[48]. However, purely relying on CCM-oriented activities provided by disease management programs may not work because there is always a quality gap when the provider has to adhere to guidelines to conduct the examinations[49]. In summary, the diabetes P4P program in Taiwan has dual characteristics that integrate extrinsic (guideline adherence) and intrinsic (CCM-oriented activities) activities at the same time, which is probably what makes the program successful[22].

Physician-led P4P (CCM component 1) and the implementation of activities based on the other 5 CCM components

We mapped the activities in the P4P program implemented in Taiwan onto the 6 components of the CCM. These 6 components are important because recent meta-analyses and systematic reviews have shown that only the integrated execution of activities corresponding to the 6 components could achieve better patient outcomes[32,50]. Another recent systematic review also demonstrated that integrating all of these components in an intervention could have greater benefits for improved patient outcomes than conducting an intervention featuring a single or some CCM components[33]. Hence, the issue of how to drive more CCM components is an important topic; the secret is perhaps found in the conclusions of Baptista *et al*[33]'s systematic review research, which highlighted the value of starting with this component (*i.e.*, leadership) and continuing to subsequently implement all the other components. Based on this research, the CCM leadership component is the key to driving the other 5 components. In addition to the incentives that are based on quality of care, part of this health system component emphasizes visible support for improvements provided by senior leadership[31,33,42]; this provider-driven leadership is important for provider engagement, and it enhances professional collaborations between providers and other medical staff (*e.g.*, health educators)[51]. Physicians, as health professional team leaders, strategically lead teams to provide coordinated care and make quality improvements[9,33].

Another example that highlights the importance of physician leadership is Wagner[52]'s revised CCM model, which was published in 1998. Interventions in health care organizations, including the delivery system design, decision support and information system categories, can help to ensure that teams are prepared and proactive, which can allow them to provide effective self-management support and access to community services to help patients be informed and activated. However, empirical research has shown that the revised CCM model still cannot address effective leadership and the robust measurement of clinical quality or effective clinical teams, which indicates the importance of starting from physician-driven leadership to lead professional teams in executing all CCM-related components[52]. Based on this research, the revised CCM model has been incorporated into the PCMH model, which emphasizes effective leadership and team building, which are essential for CCM implementation[45]; however, more evidence is needed on the effects of PCMHs in reducing patient adverse outcomes[53].

The P4P program, especially for the SCN in Taiwan, might provide physicians with the intrinsic motivation to offer provider-driven leadership to implement relevant

activities because the system meets physicians' needs to allow them to provide good-quality care and promotes providers' vision and enthusiasm to become leaders[14], which is a vital part of the successful execution of different CCM-related activities[36]. Physician-led teams also have benefits for patients because they increase patient trust and consequently promote patients' willingness to take on self-management through regular contact sessions and supervision[37].
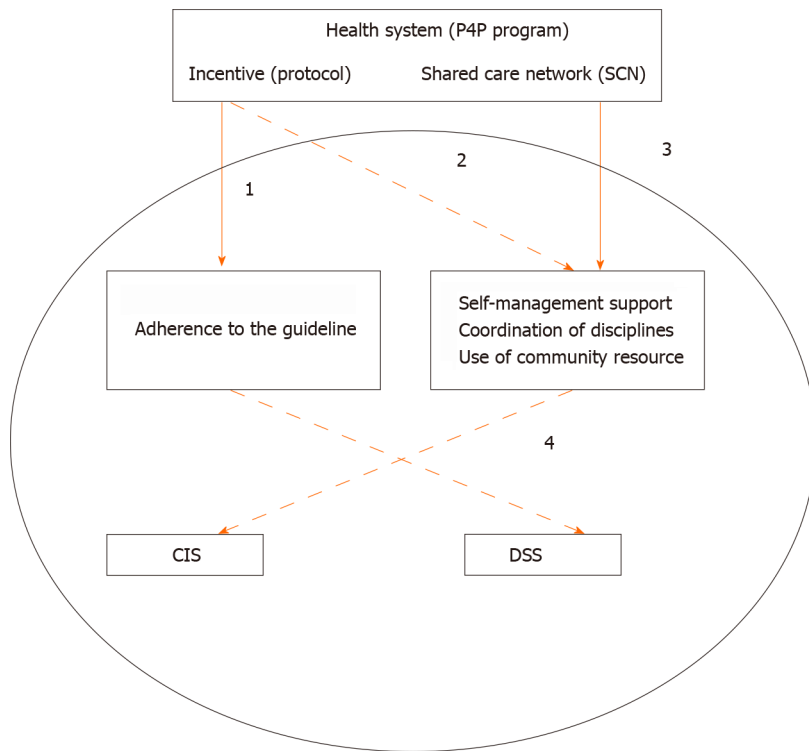
The practical framework and examples of driving other CCM components from system components can be seen in Figure 1, which starts with two health system components: incentive and SCN. In Figure 1, the top solid line represents the main target or support, and the top dashed line represents the principle provision (suggestion). The incentives mainly target guideline adherence (path 1). Other activities, including self-management support, coordination of different disciplines, and use of community resources, are only suggested in the P4P protocol (path 2). Although these activities are not the main incentive target, which means they are not paid per activity base, they are still strengthened and supported by SCN (path 3). For example, regarding coordinated care, the P4P protocol regulates team-based implementation, and the SCN provides support for team building *via* nationwide recognition. Another example is the component of self-management, where SCN provides training for coordinated care teams to boost specific caring skills, and the P4P protocol establishes the principles of patient self-management. The lower levels are the hospital-based CIS and DSS components, which are not strictly paid the incentive or supported by SCN; however, there is perhaps a spillover or induced effects on these two activities (path 4). For example, hospitals can automatically set up a DSS to promptly encourage physicians to adhere to the guidelines[34,35]. Future studies should investigate the efficiency of the framework.

CHALLENGES AND FUTURE DIRECTIONS OF THE P4P DESIGN IN THE IMPLEMENTATION OF CCM-RELATED ACTIVITIES IN TAIWAN

Taiwan's diabetes P4P framework still has room for improvement. First, approximately 30% of patients with diabetes in Taiwan were covered by the P4P program in 2010[41] and up to 43% were covered in 2015[54]. Hence, although the coverage rate of the P4P program in Taiwan approached approximately 50%, the other 50% of patients who were not covered by the program did not receive these CCM-related activities and perhaps had worse outcomes. This is probably why Taiwan is not high in the overall ranking of diabetes care across the globe[55]. However, the P4P model in Taiwan has been proven to be effective because it has the distinctive characteristics of including all CCM components. In the future, the P4P coverage rate should be steadily improved.

Second, more patients with diabetes choose to visit hospitals rather than clinics. Taiwanese citizens can visit physicians at stand-alone clinics or hospital outpatient departments for free based on their preference. Sixty percent of patients with diabetes receive care in hospitals[56]. Hospital physicians may need to treat many patients, from at least 60 to 100 or more in one day[57]. In 2018, only 8% (900/11000) of stand-alone clinics participated in the P4P program[57], and only 10% of all patients were enrolled in the P4P program in primary care (a 25% enrollment rate in primary care). Because of the limited capacity and time strain in hospital care[57], it is unlikely feasible for large hospitals (*i.e.*, tertiary hospitals) to conduct a perfect delivery system redesign, such as one that includes individualized care (case management) and tailored care based on patients' needs, which are very time-consuming. It is also unlikely for hospitals to be able to more flexibly and efficiently use community services than stand-alone clinics or practice teams since stand-alone clinics are located elsewhere in urban or rural communities. However, most of the primary care in Taiwan lacks the resources to implement activities suggested by the SCN. Since primary care may play an important role in caring for patients with diabetes[58,59], in the future, Taiwanese governments should invest more in primary care to help these facilities participate in the P4P program and have the capacity to implement CCM-related activities[60].

Third, the estimation of the incentive amount is mainly based on the number of patient visits and is not truly estimated based on the actual cost of CCM-based activities. On average, only a small amount of extra money, *i.e.*, US $100 per patient per year, can be received by the facility if the incentive amount is based on the number of patient visits[41]. Although we do not have data on the portion of P4P earnings related to the total income by physicians in Taiwan, we know that the annual amount

Figure 1 The framework of driving other chronic care model components from system components in Taiwan. P4P: Pay-for-performance; CIS: Clinical information system; DSS: Decision support system.

of the financial incentive averaged only 2%-3% of the total diabetes care expenditures (approximately US $0.4 billion]) in 2003[61], and the investment in P4P rewards was small compared to that in other countries, such as the United Kingdom, which has an indicator-based P4P system and invests large extra rewards in quality competition; for example, every GP in the United Kingdom could earn an average bonus of £77 thousand[62,63], and this approach was successful[1]. In contrast, in theory, the value creation of other P4P systems such as participatory P4P relies heavily on beneficial activities implemented for patients; more investment should be made in the core part of P4P program activities but not in measures for quality competition. If the investment is right for participatory P4P design, this design could achieve much better success. In the future, incentives should be invested directly in value-created CCM-oriented activities in Taiwan.

Fourth, the use of IT (Internet technology) to support CCM-related activities is still lacking. Systematic and meta-analyses have shown that the use of IT, such as telemedicine and mobile phones, can increase the effectiveness of diabetes patients' self-management[64]. Gee *et al*[65] proposed a future e-CCM model that consists of the use of the internet to seek health information, social networking, telehealth mobile health (mHealth), and patient portals (PRs) or patient health records (PHRs) to enhance the use of the original CCM model. The features of PRs include the tracking of patients' clinical results, proactive uptake of preventive care and screening, and suggestions for treatment strategies[66]. For example, regarding patients' self-management support, the use of PHRs, telemedicine, and mHealth could enhance patients' self-management and thus strengthen patient activation or self-perceived confidence in care[67,68] and improve patient outcomes[69]. In the future, the Taiwanese government or facilities should develop a comprehensive IT infrastructure for the e-CCM model.

CONCLUSION

We conclude that the successful characteristics of this P4P program in Taiwan include its focus on extrinsic and intrinsic incentives (*i.e.*, SCN), physician-led P4P and the implementation of activities based on the CCM components. However, due to the low rate of P4P program coverage, approximately 50% of patients with diabetes cannot enjoy the benefits of CCM-related activities or receive necessary examinations. In

addition, most of these CCM-related activities are not allotted an adequate amount of incentives, and these activities are mainly implemented in hospitals, which compared with primary care providers, are unable to execute these activities flexibly. All of these issues, as well as insufficient implementation of the e-CCM model, could hinder the advanced improvement of diabetes care in Taiwan.

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Advanced-glycation end-products axis: A contributor to the risk of severe illness from COVID-19 in diabetes patients

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Abstract

Compelling pieces of evidence derived from both clinical and experimental research has demonstrated the crucial role of the receptor for advanced-glycation end-products (RAGE) in orchestrating a plethora of proinflammatory cellular responses leading to many of the complications and end-organ damages reported in patients with diabetes mellitus (DM). During the coronavirus disease 2019 (COVID-19) pandemic, many clinical reports have pointed out that DM increases the risk of COVID-19 complications, hospitalization requirements, as well as the overall severe acute respiratory syndrome coronavirus 2 case-fatality rate. In the present review, we intend to focus on how the basal activation state of the RAGE axis in common preexisting conditions in DM patients such as endothelial dysfunction and hyperglycemia-related prothrombotic phenotype, as well as the contribution of RAGE signaling in lung inflammation, may then lead to the increased mortality risk of COVID-19 in these patients. Additionally, the cross-talk between the RAGE axis with either another severe acute respiratory syndrome coronavirus 2 receptor molecule different of angiotensin-converting enzyme 2 or the renin-angiotensin system imbalance produced by viral infection, as well as the role of this multi-ligand receptor on the obesity-associated low-grade inflammation in the higher risk for severe illness reported in diabetes patients with COVID-19, are also discussed.

Key Words: COVID-19; Diabetes mellitus; Advanced glycation; Alarmins; Advanced-glycation end-products axis; Inflammation

Core Tip: Compelling evidence support that diabetes mellitus increases the risk of coronavirus disease 2019 (COVID-19) complications, as well as the overall syndrome coronavirus 2 case-fatality. Different reports have suggested the putative involvement of several molecular mechanisms underlying this increased risk. We herein discuss the contribution of the activation of the receptor for advanced-glycation end-products axis to the higher risk for severe illness reported in diabetes patients with COVID-19.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease, where the etiological agent is a novel coronavirus, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This disease was initially detected and reported in December 2019 in Wuhan, China and then spread rapidly all over the world. This situation forced the World Health Organization to declare on January 30, 2020, the COVID-19 as a global pandemic, and thus leading humanity to face up an extraordinary challenge of a new viral disease.

Lung inflammation is the main cause of life-threatening respiratory disorders at the COVID-19 severe stage[1,2], and where lower respiratory tract symptoms and low oxygen saturation in the blood resembling acute respiratory distress syndrome (ARDS) as well as the requirement of invasive mechanical ventilation.

In addition to the lungs, SARS-CoV-2 may also infect the gastrointestinal tract, cardiovascular system, as well as central nervous system[3-5].

SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) molecule as the receptor for viral cell entry[6]. ACE2 plays an important role in renin-angiotensin system (RAS), and the imbalance between ACE/angiotensin II (Ang II)/angiotensin II receptor type 1 (AT1R) pathway and ACE2/ Ang (1–7)/Mas receptor pathway in the RAS system will lead to multisystem inflammation[7]. The activation of the AT1R by Ang II may trigger the activation of proinflammatory signals such as oxidative and nitrosative stresses, the induction of cytokines, cell adhesion molecules, as well as the activation transcription factors such nuclear factor kappa B[8-11]. Therefore, ACE2/Ang-(1-7)/Mas receptor, has been pointed out as a counter-regulator of the deleterious effects of Ang II[12].

During the pandemic, it has been shown that DM increases the risk of COVID-19 complications. Data from different studies have pointed out that increased hospitalizations, longer and repeated hospital stays as well as the overall SARS-CoV-2 case-fatality rate are significantly higher in diabetes patients who have poorly controlled glycemia when compared to patients without DM[13-16]. Although the huge amount of compelling clinical data supporting COVID-19 complications in people with diabetes, the molecular mechanisms underlying this association are not fully understood.

The receptor for advanced-glycation end-products (RAGE) was discovered as a receptor for advanced glycation endproducts (AGEs), which are accelerated formed in hyperglycemia. Afterward, RAGE emerged as a multi-ligand receptor able to interact with a diverse myriad of non-AGE ligands and being implicated in diverse chronic inflammatory states[17,18].

In the present review, we will discuss the possible contribution of the activation of the RAGE axis to the higher risk for severe illness in diabetes patients infected with COVID-19.

RAGE AXIS

RAGE was initially reported in 1992, as a membrane-associated molecule that can bind

AGEs[19]. AGEs are a heterogeneous group of molecules formed from the non-enzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids to form a freely reversible Schiff base, which spontaneously rearranges itself into an Amadori product, as is the case of the well-known hemoglobin A1c[20]. Hemoglobin A1c is an important indicator of long-term glycemic control with the ability to reflect the cumulative glycemic history of the preceding two to three months[21].

The formation of AGEs is thought to be the major cause of different diabetic complications in large part through their interactions with RAGE. Of note, AGEs may also contribute to diabetic complications through the formation of cross-links between key molecules in the basement membrane of the extracellular matrix, and thus altering the constitution of the matrix and increases stiffness[22-25].

RAGE is a single-pass transmembrane protein, which belongs to the immunoglobulin superfamily of cell surface receptors, which is now considered as a pattern recognition receptor[26]. This multi-ligand receptor is regarded as a central mediator in chronic inflammatory and immune responses[27,28].

RAGE is found in human airways with high basal levels of RAGE expressed in pulmonary tissue[29]. It is also found on vascular cells, neurons, cardiomyocytes, adipocytes, glomerular epithelial cells, or podocytes[30], as well as on pro-inflammatory and immuno-competent cells such as neutrophils, monocytes, macrophages, and T and B lymphocytes[31].

Besides AGEs, RAGE can recognize many other ligands including the alarmin high mobility group box 1 protein (HMGB1), members of the S100 protein family, glycosaminoglycans, and amyloid β peptides[32].

As a consequence of RAGE engagement by its ligands, multiple signaling pathways are triggered, including reactive oxygen species, p21ras, erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rhoGTPases, phosphoinositol-3 kinase, and the JAK/STAT pathway, having crucial downstream inflammatory consequences such as activation of nuclear factor kappa B, AP-1 and Stat-3[33].

RAGE AXIS ACTIVATION AND DIABETES COMPLICATIONS

Endogenous formation of AGEs is markedly increased in diabetes as the result of hyperglycemia and increased oxidative stress. At present, an increasing prevalence of diabetes and its complications is reported worldwide. Elevated levels of circulating AGEs are believed to play a major role in the pathogenesis of macrovascular and microvascular disease in diabetes mellitus.

Additionally, it has been demonstrated that dietary AGEs also play a major role in maintaining a high body pool of AGEs in diabetes[34].

The diabetic condition is a chronic systemic low-grade inflammation[18], and consequently, other RAGE ligands are bioavailable as is the case of some members of the S100 family and HMGB1, which can be either passively released from damaged cells or actively secreted by immune cells. A compelling body of evidence demonstrates that both AGEs and non-AGEs ligands accumulate in the plasma/serum of human subjects with diabetes[35,36].

Compelling data derived from both clinical and experimental studies support the crucial contribution of RAGE activation in vascular complications in diabetes[37].

Endothelial cells actively regulate cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. Therefore, dysfunction of the vascular endothelium is considered as an important factor in the pathogenesis of the micro-and macro-angiopathies observed in diabetes patients, and where the activation of the RAGE axis is an important contributor to this dysfunctional state[38-40].

DM has been associated with platelet hyper-reactivity, which plays a central role in the hyperglycemia-related pro-thrombotic phenotype[41,42]. In this sense, the activation of the RAGE axis has been pointed out as an important contributor to the development of a pro-thrombotic state, by its capacity to activate platelets[43,44].

COVID-19, DIABETES, RAGE AXIS, AND LUNG INJURY

DM is associated with increased disease severity and a higher risk of mortality in patients with COVID-19, who can rapidly progress to ARDS, septic shock, and multiple organ dysfunction syndrome[13-16].

Several mechanisms have been claimed for explaining the exacerbating effect of diabetes on COVID-19. These mechanisms include those directly related to hyperglycemia and the associated imbalances in pathways involved in virus entry into the cell as well as in the immune and inflammatory response. At present, the role of RAGE axis activation has been demonstrated in different animal models of ARDS and where RAGE inhibition attenuated lung injury (LI) and restored alveolar fluid clearance[45,46].

In this context, it is important to highlight that the release of the RAGE ligand HMGB1 is increased under hyperglycemic conditions[47,48], as well as the crucial role of HMGB1 in lung inflammation in diabetes[49-51].

Additionally, the contribution to LI by HMGB1-mediated RAGE signaling is well-documented in other viral diseases of the respiratory tract, as reported for the influenza virus[52].

Considering the abundance of RAGE in the lungs, the robust proinflammatory signaling triggering after the engagement, as well the relatively high expression levels in RAGE in diabetes patients[53], the activation of the RAGE axis may be an important contributor in exacerbating clinical complications in COVID-19 patients with diabetes. In this sense, it is important to highlight the contribution of RAGE axis activation in preexisting conditions such as endothelial dysfunction as well as the hyperglycemia-related prothrombotic phenotype, which increases the mortality risk of COVID-19 in DM patients.

Noteworthy, the RAGE ligand S100A12 is overexpressed in COVID-19, as recently reported[54]. This molecule is also closely related to the pathogenesis of sepsis-induced ARDS[55].

THE IMBALANCE OF RENIN-ANGIOTENSIN SYSTEM IN DIABETES

The association of the RAS with the endocrine system is particularly illustrated by the prominent role of Ang II in diabetes and metabolic syndrome. RAS has been extensively described to be involved in the onset and progress of hypertension, retinopathy, nephropathy, and cardiovascular disease in DM patients. RAS is considered an important pharmacological target in the management of micro-and macrovascular complications for these patients[56-59].

Of particular importance, individuals with diabetes have a reduced ACE2 expression. This enzyme is found in multiple organs including the lungs. ACE2 plays an important role in the RAS, and the imbalance between ACE/Ang II/AT1R pathway and ACE2/Ang (1–7)/ Mas receptor pathway in the RAS system will lead to multisystem inflammation. This reduced expression confers to individuals with diabetes an increased risk of severe LI as well as ARDS if infected by COVID-19[60].

SARS-CoV-2 INFECTION, RENIN-ANGIOTENSIN SYSTEM IMBALANCE, AND THE RAGE AXIS

As already mentioned SARS-CoV-2 uses ACE2 molecule as the receptor for viral cell entry[6]. ACE2 is a key counter-regulatory element in the pathway of the renin-angiotensin system, which acts to opposite the actions of Ang II by generating Ang-(1–7), and thus reducing inflammation and fibrosis and mitigate end-organ damage[61].

Strikingly, SARS-CoV-2 hijacks ACE2 to invade and damage cells, downregulating ACE2, reducing its protective effects, and exacerbating injurious Ang II effects[62].

Considering the facts that diabetes patients have a reduced expression of ACE-2, as well as the capacity of SARS-CoV-2 to hijacks ACE2, ACE2 exhaustion will be produced in patients with diabetes during infection and, thus reducing its capacity to fully function as a counterbalancing element of RAS through the ACE2/Ang-(1-7)/mas receptor pathway.

Decades of research have demonstrated that the activation of ATR1 by Ang II, triggers a robust inflammatory response involving the recruitment and activation of inflammatory cells, as well as apoptosis of both alveolar epithelial cells and pulmonary microvascular endothelial cells, and consequently, a marked increased microvascular permeability and loss of epithelial and endothelial integrity[63].

RAGE axis is an important contributor to the pathophysiology of lung inflammation because the use of different inhibition strategies can increase arterial oxygenation,

reduce alveolar inflammation, and improve lung damage in acute lung inflammation[46,64].

Strikingly, a novel ligand-independent mechanism for RAGE transactivation has been recently reported to occur following activation of the AT1R by Ang II and thus leading to nuclear factor kappa B dependent expression of pro-inflammatory mediators[65]. This novel mechanism is expected to continuously fuel the lung inflammatory environment in diabetes patients during SARS-CoV-2 infection, considering both the high expression of RAGE and the reduced levels of ACE-2 in the lungs[66].

SARS-CoV-2, CD-147, AND THE RAGE AXIS

Increased infiltration and accumulation of macrophages is a common process in many of the complications of diabetes patients[67].

CD147, originally described in tumor cells, is a highly glycosylated 58-kDa transmembrane protein belonging to the immunoglobulin superfamily and also known as extracellular matrix metalloproteinase functions as a matrix metalloproteinases (MMPs) inducer, predominantly MMP-2 and MMP-9. Of note, the expression of this protein is markedly increased by AGEs by a RAGE-dependent mechanism[68].

Degradation of protein components in the alveolar epithelial–endothelial unit by both MMP-2 and MMP-9 is considered a central process in the pathogenesis of ALI/ARDS[69-71]. Strikingly, SARS-CoV-2 spike protein may bind also to CD147 glycoprotein[72], and thus mediating viral invasion. Due to the high expression levels of this protein in diabetes, this condition may then increase the accessibility of virus to tissue in patients with diabetes. A recent report demonstrates the Meplazumab, a humanized anti-CD147 antibody efficiently improved the recovery of patients with SARS-CoV-2 pneumonia with a favorable safety profile[73].

SARS-CoV-2, THROMBOTIC MICROANGIOPATHY, AND RAGE AXIS

Thrombotic microangiopathy is reported as a frequent event in COVID-19[74]. In patients with diabetes, endothelial dysfunction is a very common condition, and events such as enhanced vasoconstriction, platelet hyperactivity and thrombus formation are activated due to the metabolic milieu, and where the activation of the RAGE axis is continuously fueled by hyperglycemia, insulin resistance, and the oxidative stress seen in diabetes[75]. Noteworthy, platelets can be activated by a RAGE-dependent mechanism[43].

The dysfunctional state of the endothelium is linked to an impairment of nitric oxide production and activity, which may then affect not only the vasodilator tone and platelet activity but also the recruitment of endothelial progenitor cells, which directly contribute to the homeostasis and repair of the endothelial layer in blood vessels[76-78].

Very recently, clinical findings suggest that SARS-CoV-2 infection facilitates the induction of endotheliitis in several organs as a direct consequence of viral infection[79]. However, these data have generated controversy about the nature of the viral-type particles reported because of endoplasmatic reticulum may mimic SARS-CoV-2 particles on electron microscopy[80,81]. Additionally, other pieces of evidence show the absence of viral ribonucleic acid inside endothelial cells, suggesting that indirect effects rather than direct viral infection might trigger endothelial damage[82]. On the other hand, SARS-CoV-2 spike protein may bind also to CD147 glycoprotein which is upregulated by hyperglycemia and by RAGE activation[68]. CD147 expression is significantly upregulated in activated endothelial cells[83]. Therefore, these findings raise the intriguing possibility that RAGE activation may play a role also in viral invasion to host cells.

The activation of the RAGE axis has been widely documented to be crucial to prime proinflammatory mechanisms and rendering endothelial cells into an activation state and thereby amplifying proinflammatory mechanisms in many chronic inflammatory disorders[84-86]. Thus, preexisting blood vessel damage may put people with COVID-19 at heightened risk of complications from the infection.

A dysfunctional endothelium as observed in diabetes, leading to detrimental shifts in the vascular equilibrium towards vasoconstriction, inflammation, and a procoagulant state resulting in thrombosis, constitute a much more proper condition to fuel inflammation in the blood vessel wall and then putting diabetes patients with COVID-19 at heightened risk of complications from the infection.

SARS-CoV-2, OBESITY, DM-2, AND RAGE AXIS

More than 90% of patients with type 2 diabetes have obesity or overweight[87]. In the context of the COVID-19 outbreak, many reports highlight that obesity and type 2 diabetes as comorbidities of SARS development in COVID-19 patients[88-90].

Both obesity and type 2 diabetes are associated with a chronic low-grade inflammatory state, and this particular basal state could then aggravate the inflammatory response to SARS-CoV-2 infection observed in severe COVID-19 cases.

In this context, there are shreds of evidence suggesting a key role of RAGE axis activation in fat tissue inflammation, and thus contributing to the obesity-associated low-grade inflammation, as well as to the reported dysregulation of adipokines[91,92].

Furthermore, many RAGE ligands such as AGEs, HMGB1, and S100/calgranulins, accumulate in adipose tissue in many models of obesity as well as in obese subjects[93-96], where they can trigger a robust proinflammatory secretion profile, which in turn, establishes a vicious loop, and thus rendering more inflammation[97].

The low-grade inflammation in adipose tissue is characterized, in addition to the robust secretion of proinflammatory cytokines, by the recruitment of leukocytes, mainly macrophages in this tissue. The accumulation of macrophage into adipose tissue correlates to both the degree of adiposity as well as the production of monocyte chemoattractant protein-1, which in turn, recruit more macrophages and thereby promote the chronicity of inflammation[98].

Furthermore, macrophages infiltrated in adipose tissue undergo a polarization process towards a spectrum of different phenotypes where two extremes are represented by the classically activated type 1 macrophages and the alternative activated type-2 macrophages[99]. Noteworthy, RAGE ligands accumulation and macrophage type 1 macrophages polarization are much more prevalent in perivascular adipose tissues[100] and thus, adding more inflammation to the vascular system.

During this pandemic, some alerts have been raised on side effects of some widely used drugs on diabetic COVID-19 patients, particularly lactic acidosis and ketoacidosis (DKA) for metformin and sodium-glucose cotransporter 2 inhibitors, respectively[101,102].

The RAGE axis has been recently suggested to be a crucial contributor to the acute inflammatory insult during the medical crisis and treatment of DKA and thus acting as a constant source of subclinical inflammation leading to chronic diabetic vascular complications, including those of the heart[103].

Additionally, 3-deoxyglucosone is significantly elevated before and during the treatment of DKA[104]. 3-Deoxyglucosone is a dicarbonyl species that may lead to the formation of AGEs, and then fueling inflammation by RAGE engagement[105].

One mechanism by which metformin increases plasma lactate levels relates to the inhibition of mitochondrial respiration responsible for lactate removal[106,107], which correlate with the inhibition of mitochondrial oxidative phosphorylation[108].

The activation of the RAGE axis is known to increase cytosolic reactive oxygen species production which, in turn, facilitates mitochondrial superoxide production in hyperglycemic environments, and thus rendering a mitochondrial dysfunctional state[109,110]. This particular dysfunctional state could be a particular life-threatening condition in diabetic COVID-19 patients[111].

CONCLUSION

At present, a compelling body of evidence supports the crucial role of the RAGE axis in the pathophysiology of diabetes, being a key contributor in the onset and sustainment of low-grade and chronic inflammation state observed in patients with diabetes, and consequently, marked impairment of endothelial functions. Thus, this basal hyper-activated state of the RAGE axis, as occurs in diabetes patients may represent a crucial element in many clinical complications in diabetes patients who develop COVID-19 (Figure 1).

Furthermore, the novel ligand-independent transactivation of the RAGE axis by AT1R/Ang II further strengthens the hyperactivation state of the axis and consequently, fueling a robust pro-inflammatory environment particularly in the low respiratory tract, where the high expression of RAGE and AT1R receptors plays an essential role in the pathophysiology of the lung inflammation observed in those diabetic patients.

Figure 1 The chronic and low-grade inflammatory state preexisting in diabetes patients as well as in one of the most frequent co-morbidities observed in diabetes seems to be particularly exacerbated in coronavirus disease 2019 patients with diabetes. The receptor for advanced-glycation end-products axis hyper- activation, either by ligand-dependent or cognate-ligand independent mechanisms, is emerging as crucial contributor to this huge inflammatory response leading to acute respiratory distress syndrome, endotheliitis and thrombotic complications. ARDS: Acute respiratory distress syndrome; AT1R: Angiotensin II receptor type 1; COVID-19: Coronavirus disease 2019; RAGE: Receptor for advanced-glycation end-products; RAS: Renin-angiotensin system.

In summary, in light of what is known about the poor clinical outcomes of diabetic patients who develop COVID-19, the RAGE axis seems to be one of the key players in the enhanced inflammatory response and the high mortality rates of these patients. While the precise mechanisms by which the RAGE axis activation contributes to the higher risk of severe illness in diabetes patients infected with SARS-CoV-2 remain to be fully understood, it is important to strengthen future clinical research in this area.

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Current advances in using tolerogenic dendritic cells as a therapeutic alternative in the treatment of type 1 diabetes

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing β-cells of the pancreatic islets by autoreactive T cells, leading to high blood glucose levels and severe long-term complications. The typical treatment indicated in T1D is exogenous insulin administration, which controls glucose levels; however, it does not stop the autoimmune process. Various strategies have been implemented aimed at stopping β-cell destruction, such as cellular therapy. Dendritic cells (DCs) as an alternative in cellular therapy have gained great interest for autoimmune disease therapy due to their plasticity to acquire immunoregulatory properties both *in vivo* and *in vitro*, performing functions such as anti-inflammatory cytokine secretion and suppression of autoreactive lymphocytes, which are dependent of their tolerogenic phenotype, displayed by features such as semimature phenotype, low surface expression of stimulatory molecules to prime T cells, as well as the elevated expression of inhibitory markers. DCs may be obtained and propagated easily in optimal amounts from peripheral blood or bone marrow precursors, such as monocytes or hematopoietic stem cells, respectively; therefore, various protocols have been established for tolerogenic (tol)DCs manufacturing for therapeutic research in the treatment of T1D. In this review, we address the current advances in the use of tolDCs for T1D therapy, encompassing protocols for their manufacturing, the data obtained from preclinical studies carried out, and the status of clinical research evaluating the safety, feasibility, and effectiveness of tolDCs.

Key Words: Type 1 diabetes; Dendritic cells; Autoimmunity; Immune tolerance; Cell therapy; Immunotherapy

Core Tip: Autoimmunity in type 1 diabetes (T1D) is severe and leads to pancreatic dysfunction; therefore, therapies that can lessen this process are required. Cell therapy with tolerogenic dendritic cells (tolDCs) is a promising strategy. Various protocols have been implemented for tolDC generation, using stimuli such as cytokines, growth factors, and drugs. These cells are also subjected to treatments with antisense oligonucleotides, liposomes, toll-like receptor ligands, and peptides of the pancreatic islets, for optimization as T1D immunotherapy. Preclinical and clinical trials have demonstrated effectiveness of tolDC-based therapy. This review aims to give a detailed understanding of current advances in tolDC-based T1D treatment.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the dysfunction and destruction of insulin-producing β-cells in the pancreatic islets of Langerhans[1,2]. Genetic susceptibility contributes to the loss tolerance of β-cells antigens, such as insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated-2 autoantibodies, and ZnT8 by autoreactive CD4+ and CD8+ T cells, leading to islet destruction, insulin deficiency, and elevated blood glucose levels[3-7].

Some current therapeutic strategies for T1D treatment include the exogenous insulin replacement therapy and the use of immunosuppressive drugs, leading to the amelioration of several aspects inside the pathology, but not the causal factors[8], as well as other different conditions[9]. Furthermore, serious side effects, like chronic infections or malignant transformation, may be driven by the use of immunosuppressive drugs. Therefore, alternative therapeutic strategies are necessary to reach the maintenance, restoration, or induction of autoantigen-specific immunological tolerance. In this line, cellular immunotherapy is emerging as a promising approach for the treatment of a T1D, inside which the use of tolerogenic dendritic cells (tolDCs) has attracted special attention[10].

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) specialized in the initiation of both immunogenic and tolerogenic response[11]. For immunogenic activities, DCs mature in response to inflammatory stimuli. During this maturation process, DCs markedly increase the expression of major histocompatibility complex (MHC)-peptide complexes and costimulatory molecules, secrete a wide variety of pro-inflammatory [tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, and IL-6] and immunomodulatory [interferon (IFN)-α, -β and -γ, and IL-12] cytokines, augmenting their ability to prime T cells[12,13]. On the other hand, for tolerogenic functions, both in central and peripheral tolerance[13], DCs acquire tolerogenic properties, named as "tolerogenic DCs or tolDC" with the capacity to regulate potential harmful adaptive responses[14,15]. Such tolerogenic properties result from their low capability to stimulate T cells, their high secretion of immunoregulatory factors such as anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF) -β], indolamine 2,3-dioxygenase, and the expression of surface inhibitors like programmed death-ligand 1 (PD-L1)[15-17].

In addition, T1D-associated genetic factors are expressed in DCs affecting their tolerogenic properties *in vivo*[18], and the hyperglycemic state, as well as the control degree of patients, may affect the optimal regulatory functions of tolDCs[19]; by this reason, the immunoregulatory characteristics of tolDCs have made them potential tools for therapeutic research for T1D[20]. In this line, several protocols have harnessed DC plasticity to respond to external immunomodulatory agents modifying their phenotype, cytokine profile, and stimulatory ability, with the aim of developing manufacturing of tolDCs as a method to control T cell-mediated immunopathologic processes occurring in T1D and, simultaneously, as a replacement mechanism that might lead to the restoration of innate tolerance control. However, owing to several aspects concerning the efficacy, safety, and feasibility, essentially about the per-

formance of the protocols to obtain tolDC with stable tolerogenic phenotype, the therapeutic use of tolDCs requires further analysis. This review approaches advances in the investigation of protocols for tolDC manufacturing, as well as the underlying tolerogenic mechanisms described from their pre-clinical and clinical use in T1D.

STRATEGIES FOR TOLDCS MANUFACTURING: ALTERNATIVELY AND *EX VIVO* GENERATED

DCs endowed with tolerogenic properties have been widely characterized for presenting a semimature state accompanied with high antigen uptake ability, a reduced antigen presentation capability owing to an attenuated antigen processing, and a reduced expression of MHC-II/MHC-I and costimulatory molecules, which in turn limits their competence to stimulate naïve or effector/memory T cells. Additionally, tolDCs produce reduced o null levels of pro-inflammatory cytokines such as IL-12p70; in contrast, they secrete a high level of anti-inflammatory cytokine like IL-10[21]. Nevertheless, the expression of some markers may be variable depending on the protocols used for tolDC generation. Concerning their functionality, tolDCs avoid the activation of autoreactive T cells by inducing various tolerance mechanisms, such as apoptosis, skewing phenotype, anergy, and expansion or induction of regulatory T cells (Tregs)[13,21]; a general view of tolerogenic features of tolDCs is shown in Figure 1.

Several protocols have been established for the differentiation and propagation of tolDCs. In humans, tolDCs are generated from peripheral blood monocytes (alternatively generated tolDC) and in murine models from bone marrow progenitors (*ex vivo* generated tolDC)[22,23]. The manufacturing of tolDC is carried out by exposing the cells to growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) and cytokines like IL-4, which induce differentiation to immature DC, and the simultaneous use of immunomodulatory agents such as anti-inflammatory cytokines (IL-10 and/or TGF-β) or pharmacological agents (dexamethasone, rapamy-cin, and vitamin D3) that allow obtainment of tolerogenic properties[24-27] (Table 1 and Figure 2).

At first, typical protocols for alternative and *ex vivo* tolDC generation are carried out in the presence of GM-CSF alone or plus IL-4 (GM-CSF/IL-4). GM-CSF is important in the functional regulation of DCs; studies in a murine model revealed that generation of bone marrow-derived DCs with low concentrations of GM-CSF possess an immature phenotype, resistant to maturation and restore T cell tolerance *in vivo* and *in vitro*[28]. It has been reported that GM-CSF provides protection against diabetes in non-obese diabetic (NOD) mice. DCs of these GM-CSF-protected mice express low levels of MHC-II, CD80, and CD86, produce IL-10, and are less effective in stimulating diabetogenic CD8+ T cells[29]. Additionally, DCs that are treated to express IL-4 can delay or prevent the onset of autoimmune diabetes in NOD mice, maintaining stable glucose levels for a long time[30]. Strikingly, it has been documented that GM-CSF/IL-4 combination synergistically improved the regulatory roles of DCs, demonstrating optimal prevention of diabetes in NOD mice[22,31].

Besides using GM-CSF/IL-4, tolDCs have been generated *in vitro* by adding immunomodulatory agents during the process of differentiation. Our research group demonstrated that tolDC inducing antigen-specific tolerance may be generated when they are alternatively differentiated in the presence of cytokines such as IL-10/TGF-β1 together, displaying enhanced efficiency to generate anergy and Tregs[24]. These tolDC displayed lower expression of CD40, enhanced endocytic ability, increased secretion of IL-10 and prostaglandin E, and lowered secretion of IL-12 and IL-23. On the other hand, tolDCs have also been generated by only using a suppressive modulator like IL-10[24,32] or TGF-β[33]; such cells show increased secretion of IL-10 and IL-6, reduced IL-12p70 production, and a semi-mature phenotype demonstrated by intermediate expression of CD80, CD86, CD40, CD83, and MCH-II. Another protocol for tolDC generation is GM-CSF/IL-10. TolDC obtained by this route modulate the autoimmunity in a specific form when they are differentiated in the presence of autologous serum[34]. It has also been demonstrated that tolDC (GM-CSF/IL-10) in animal models of T1D suppress insulitis and spontaneous diabetes in NOD mice. These results suggest that IL-10-treated DC acquire tolerogenic characteristics and induce tolerance in pancreatic islets in a non-antigen-specific way[35]. Likewise, DCs induced by TGF-β display tolerogenic phenotype and func-tions. The addition of these TGF-β-treated tolDC to grafted islets led to graft survival in autoimmune diabetic recipient mice[33].

Table 1 Tolerogenic dendritic cells manufacturing for type 1 diabetes therapy

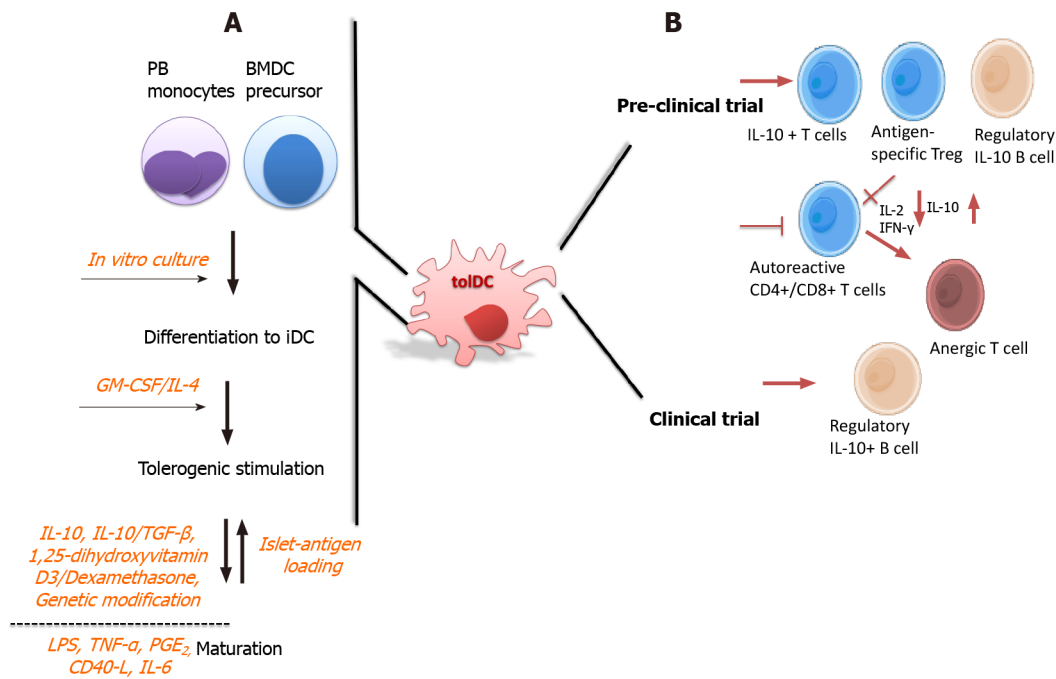| Protocol	Treatment	DC phenotype	Therapeutic effects in T1D	Ref.
GM-CSF	Apoptotic bodies-loaded	↓ Costimulatory molecules (CD40, CD86); ↓ IL-6; ↓ TNF-α	Reduces disease incidence in NOD mice. Reduces insulitis	Marin-Gallen *et al*[62], 2010
	Liposomes-loaded	↑ TIM4, CD36; ↓ MHC-II; ↓ Costimulatory molecules (CD40, CD86); ↑ CCR7, CCR2; ↑ DC-SING; ↓ IL-6; ↑ Anti-inflammatory cytokines (IL-10, TGF-β1)	Decreases CD8^{+} T cell proliferation. Reduces disease incidence in NOD mice. Reduces insulitis	Pujol-Autonell *et al*[64], 2015
GM-CSF/IL-4	Antisense oligonucleotides	↓ Costimulatory molecules (CD40, CD80, CD86); ↓ NO; ↓ TNF-α, IL-12p70	Prevents diabetes in NOD mice. Reduces insulitis. Promotes Tregs. Increases B cells. Suppresses T cells proliferation: Clinicaltrials.gov identifier: NCT00445913; Clinicaltrials.gov identifier: NCT02354911	Machen *et al*[50], 2004
				Di Caro *et al*[51], 2014
				Di Caro *et al*[52], 2012
				Phillips *et al*[53], 2008
				Giannoukakis *et al* [54], 2011
				NIH[68], 2007
				NIH[69], 2015
	Antigen-loaded: Proinsulin	Tolerogenic phenotype (not specifically described)	Delays or halts progressive destruction of β-cell and loss function. -Clinicaltrials.gov identifier: NCT04590872	Nikolic *et al*[70], 2020
				FACT[71]
	Liposomes-loaded	↓ Costimulatory molecules (CD40, CD86); ↑ PDL1 expression; ↑ VEGF secretion	Arrests autoimmunity in the model of experimental diabetes	Rodriguez-Fernandez *et al*[61], 2019
				Rodriguez-Fernandez *et al*[63], 2018
	TLR´s ligand: 1Z1	↑ PD-L1; ↑ IRAK-M; Minimum increases of MHC-II, CD40, CD80, CD83, CD86	Suppresses T cell activation and proliferation. Delays insulitis in NOD mice	Kim *et al*[67], 2012
GM-CSF/IL-10		↓ Costimulatory molecules; ↓ IL-12, IL-23, IL-6; ↑ IL-10	Reduces insulitis. Prevents spontaneous diabetes in murine models. Induces Tregs. Induces hyporesponsiveness of T cells. Inhibits T cells proliferation	Haase *et al*[34], 2005
				Tai *et al*[35], 2011
GM-CSF/IL-4 + IL-10 or TGF-β		Intermediate expression of MHC-II, CD40, CD80, CD86, CD83; ↓ IL-12p70, IL-23, TNF-α; ↑ IL-10; ↑ IL-6; ↑ PD-L1	Decreases T cells infiltration. Reduces T cells proliferation. Induces Tregs. Prolongs the survival of syngeneic Islet graft in NOD mice	Torres-Aguilar *et al*[24], 2010
				Boks *et al*[32], 2012
				Thomas *et al*[33], 2013
GM-CSF/IL-4 + IL-10/TGF-β	Antigen-loaded: Insulin; GAD65	↑ CD1a; ↓ Costimulatory molecules (CD40, CD86); ↓ CD83; ↓ MHC-II; ↓ IL-12; ↓ IL-23; ↑ PGE	Suppresses effector/memory T cells. Induces T cells anergy. Induces Tregs. Induces IL-10 production by T cells. Suppresses T cells proliferation. Induces hyporesponsiveness of T cells	Torres-Aguilar *et al*[44], 2010
				Segovia-Gamboa *et al*[58], 2014
GM-CSF/IL-4 + Vitamin D/Dexamethasone	Antigen-loaded: -Proinsulin	↓ MHC-II; ↓ IFN-γ; ↓ CD86; ↑ IL-10; ↑ PD-L1	Controls autoimmunity. Induces Tregs. Inhibits effector T cells. Eliminates CD8^{+} T cells	Suwandi *et al*[55], 2020
				Gibson *et al*[56], 2015
	-GAD65	↓ Costimulatory molecules (CD40, CD86); ↓ CD83; ↓ MHC-II; ↑ CD14; ↑ TLR-2; ↑ PD-L1; ↑ IL-10; ↓ IL-6, TNF-, IL-23, IL-12p70	Decreases Th1/Th17 responses. Suppresses antigen-specific T cell activation and proliferation. Prevents onset diabetes in NOD-SCID mice. Decreases IFN-γ production by T cells	Phillips *et al*[20], 2017
				Funda *et al*[57], 2018
GM-CSF/IL-4 + Rapamycin		↓ Costimulatory molecules (CD40, CD80); ↓ IL-6, IL-23; ↑ PD-L1	Induces Tregs. Inhibits T cell proliferation. Reduces Th17 cells	Boks *et al*[32], 2012
				Navarro-Barriuso

	et al[39], 2018

T1D: Type 1 diabetes; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; NOD: Non-obese diabetic; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MHC: Major histocompatibility complex; TGF-β1: Transforming growth factor-β1; VEGF: Vascular endothelial-derived growth factor; TLR: Toll-like receptor; PD-L1: Programmed death-ligand 1; IRAK-M: IL-1 receptor-associated kinase M; PGE: Prostaglandin E; IFN-γ: Interferon-γ; DC: Dendritic cell; Treg: Regulatory T cell; GAD65: Glutamic acid decarboxylase 65.

Figure 1 Phenotypic and functional hallmarks describing the immunobiology of the tolerogenic state of dendritic cells. Tolerogenic dendritic cells (tolDCs) display a semimature state with high antigen uptake capability and bear low/intermediate surface levels of factors essential for T cell priming. In contrast, tolDCs bear high surface levels of inhibitory markers, allowing them to inhibit autoreactive T cells. Further, tolDCs display reduced secretion of inflammatory/immunomodulatory agents accompanied by the high secretion of anti-inflammatory/suppressive modulators. All those features are essential for inducing specific tolerance for self, microbiome, and environmental derived antigens by mechanisms such as anergy, deletion, phenotype skewing, and/or expansion of regulatory T cells. Additionally, tolDCs display optimal migratory capability, which has been documented to be essential to inducing periphery tolerance *in vivo*. HLA: Human leukocyte antigen; IFN: Interferon; IL: Interleukin; TGF: Transforming growth factor; tolDC: Tolerogenic dendritic cells; TNF: Tumor necrosis factor; MHC: Major histocompatibility complex; PD-L1: Programmed death-ligand 1.

Additionally, one of the major drugs used to induce tolDC differentiation *in vitro* is the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3. These tolDC display different morphological features than immunogenic DCs, showing lower expression of costimulatory molecules and high CD11c and DC-SIGN expression, confirming their semi-mature phenotype, with an increased expression of IL-10 and inhibitory molecules like PD-L1[36-39]. Vitamin D3 and dexamethasone combination generate tolDC characterized by a low expression of MHC-II, the costimulatory molecules CD40 and CD86, and the maturation marker CD83, as well as low levels of IL-12p70[40]. Nevertheless, a systematic comparative analysis of tolDC generated with vitamin D3, IL-10, dexamethasone, TGF-β, or rapamycin showed that IL-10-generated tolDCs are optimal for functional Treg induction, which display strong suppression activity[32]. Likewise, several agents of different nature have been used for tolDC generation; such agents encompass tissue-derived factors, cytokines, some pathogen-derived antigens, and pharmacological molecules[41,42].

One significant aspect taking special attention is the notion that tolDCs might not be advisable for clinical research, because the *in vivo* permanence of their phenotype and tolerogenic functions might not be guaranteed, especially when they reach tissues with chronic inflammation in conditions such as T1D[43]. In this line, several investigations have addressed protocols that allow obtaining functionally stable tolDC to keep their regulatory properties under pro-inflammatory environments. These protocols include addition of maturation stimuli, such as lipopolysaccharide, TNF-α, prostaglandin E$_2$, CD40-L, or IL-6 between others[43-45], during or after the tolerogenic stimuli. Although the rising idea about the maturation state is not necessarily a fully distinguishing feature of immunogenic activity on DCs, a mature state is not opposed to their tolerogenic activity either[46]. Besides the stable tolerogenic phenotype, a mature state of tolDC may optimize some features for optimal regulatory mechanisms *in vivo*, such as their migratory capability, which is required to promote T cells into regulatory control. Moreover, such migratory capability has been considered as a pivotal

Figure 2 Tolerogenic dendritic cells in type 1 diabetes therapy: manufacturing and tolerogenic mechanisms described in preclinical and clinical trials. A: Tolerogenic dendritic cells (tolDCs) are alternatively generated from peripheral blood monocytes, or bone marrow precursors, which are subjected in culture with sequentially stimulation processes. Immature DC differentiation is firstly generated with growth factors, which in turn, owing to their plasticity, are subjected to tolerogenic stimulation with immunomodulatory agents to obtain tolDCs. Besides, some protocols perform the manufacturing with additional maturation stimuli, such as lipopolysaccharide or tumor necrosis factor-α previous to or after the tolerogenic stimulus to obtain stable tolDCs; B: According to their regulatory mechanism, tolDCs may induce an increased frequency of interleukin (IL)-10-expressing T cells and expand the antigen-specific regulatory T cell population, which show optimal suppressive activity; further, tolDCs reduce the activation and proliferation of autoreactive naïve and memory CD4+ and CD8+ T cells, otherwise becoming anergic. Additionally, the regulatory roles of tolDCs also reach B cells, since a high level of regulatory B cells expressing IL-10 are expanded by tolDCs, which are associated to a protective role in type 1 diabetes, being the only described immunoregulatory mechanism obtained from a clinical trial. BMDC: Bone marrow-derived dendritic cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; PGE$_2$: Prostaglandin E$_2$; PB: Peripheral blood; IL: Interleukin; tolDC: Tolerogenic dendritic cell; TGF: Transforming growth factor; LPS: Lipopolysaccharide.

tolerogenic feature of DCs[32,45].

Preclinical assays: In vitro and in vivo studies evaluating the promising tolDC application for T1D therapy

Several studies have revealed a diversity of regulatory mechanisms employed by tolDCs both *in vitro* and in animal models. Such mechanisms differ according to the type of generated tolDC. Hence, this evidence has prompted their use for further clinical research (Figure 2).

According to *in vitro* analysis, IL-10-generated tolDCs have been documented to be optimal to induce Tregs with strong suppressive activity[32,45]. Such tolDCs are resistant to inflammatory conditions and exhibit strong migratory capacity toward the secondary lymphoid organ chemokine CCL21, allowing an optimal migratory capability to induce T cell regulatory actions. In line with their regulatory activity on autoreactive T cells, *ex vivo* 1,25-dihydroxyvitamin D3-generated tolDCs derived from diabetes-prone (NOD) mice decrease the proliferation and activation of autoreactive CD4+ T cells *in vitro*. Further, these tolDCs are optimal to induce increased IL-10 expression in T cells and may expand the CD25+ Foxp3+ T cell population[47]. Regarding CD8+ T cells' activity as an independent risk factor governing the detrimental destruction of insulin-producing β-cells by their cytotoxic role[48], vitamin D3/dexamethasone-modulated DCs (Combi-DCs) loaded with human leukocyte antigen class I epitopes were described with the capability to impede priming of autoreactive naïve CD8+ T cells and to reduce memory CD8+ T cells[36].

Other studies have displayed the effective regulatory role of tolDCs on T cells, but data illustrating further tolerogenic mechanisms are scarce. Additionally, some studies have shown that inhibitory roles of tolDC are not limited to T cells, since such effects may even reach B cells by increasing their frequency. *Ex vivo* generated tolDCs appear to be capable of inducing the expansion of regulatory B cells, displaying an IL-10-dependent suppressive effect on T cells through the proliferation of preexisting IL-10-

expressing B cells as well as by differentiation of their precursors. This mechanism performed by tolDCs is mediated in a retinoic acid-dependent manner, favoring the FoxP3^{+} Treg differentiation[49]. These findings describe a novel mechanism of tolDCs exerting their regulatory mechanism on other cellular entities different than Tregs.

Regarding the *in vivo* analysis, the NOD mouse model is a well-established approach extensively used to investigate several aspects of the molecular and cellular mechanisms underlying T1D as well as to evaluate therapeutic agents. TolDC have been shown to prevent and reverse T1D in the NOD mouse model. *Ex vivo* generated tolDCs with impaired costimulatory capability, delay or revert new-onset hyperglycemia for the long-term, increasing the expansion of Tregs[50,51]. Additionally, an increased number of regulatory B cells (Breg) expressing higher levels of IL-10 has been obtained from NOD mice. Such Bregs resulted from the conversion of precursor B cells into IL-10-expressing cells, being, in this way, involved in the mechanism of tolerogenic reversal of T1D by tolDC[51]. Moreover, *ex vivo* 1,25-dihydroxyvitamin D3-generated tolDCs transferred into NOD severe combined immunodeficiency mice exhibited the capability to dampen autoreactive T cell proliferation in pancreatic draining lymph nodes. This action probably might be an effect of the functional migratory capability of tolDC, since these tolDCs exhibited optimal homing to the pancreas in adult NOD-SCID mice[47].

Nowadays, there is a diversity of preclinical trial protocols for tolDC aimed at T1D immunotherapy. During or after differentiation and propagation of tolDC, different strategies have been implemented in order to improve the functional capability of tolDC. Within those protocols, there is the use of antisense oligonucleotides targeting the expression of costimulatory molecules, tolDCs pulsed or loaded with antigens for a specific antigen immune response, liposomes or apoptotic bodies, and the use of Toll-like receptor (TLR) ligands.

Antisense oligonucleotides: TolDCs may be obtained by genetic modification, including transference or silencing of selected genes through several approaches, with the aim to modulate their maturation. In T1D immunopathogenesis there are active DCs favoring the increase of costimulatory molecules to realize immunogenic functions; for this reason, a protocol was implemented to negatively regulate their expression through *ex vivo* treatment of immature DCs from NOD mice with a mixture of antisense oligonucleotides targeting the CD40, CD80, and CD86 transcripts[50].

The single administration of these tolDCs promotes a higher prevalence of Tregs, conferring protection against T1D[50]. These phosphorothioate-modified antisense oligonucleotides confer tolerogenic properties to cells and prevent T1D in NOD mice, therefore, some research groups proposed developing microspheres "DC populations targeting" of the three antisense oligonucleotides, and this broadens the perspective towards a possible vaccine of treated tolDCs[52,53]. This method of antisense oligonucleotides has allowed launching of the first phase I study of autologous tolDC administration in T1D therapeutics[54].

Antigen-loaded tolDCs: Immunotherapies with tolDCs can be performed with antigen-loaded or -unloaded cells; both methods have shown results preventing T1D. However, the antigen-loaded methods promise being more feasible, owing to these, should specifically inhibit the action of autoreactive T cells, thereby allowing a tolerance restoration to self-antigens and avoiding general immunosuppression.

Proinsulin, insulin, and GAD65 are some target autoantigens involved in T1D development and, hence, utilized to load tolDCs. Vitamin D3/dexamethasone-generated and proinsulin-loaded tolDCs induce antigen-specific Tregs with various phenotypes *in vitro*, expressing regulatory markers, such as Lag-3, CD161, and inducible co-stimulator, and effectively suppress effector CD8^{+} and CD4^{+} T cells[55]. Likewise, in a humanized mouse model of proinsulin autoimmunity, the administration of proinsulin in vitamin D3-generated tolDCs may control the autoimmunity *via* IL-10 production[56]. Controversially, in another study, the authors tested the efficacy of vitamin D2/dexamethasone-generated GAD65-loaded tolDCs to prevent the adoptive transfer of diabetes by diabetogenic splenocytes to NOD-SCID receptor mice. However, in this study the GAD65-loaded tolDCs decrease the protective effect against disease in T1D, compared to tolDCs without antigen-loading[57].

The evidence that the metabolic control of T1D individuals affects the functionality of tolDCs takes special relevance in tolDC-based strategies. Alternatively generated tolDCs modulated with vitamin D2/dexamethasone were loaded with the antigen GAD65 from well- and deficient-controlled T1D patients. Results showed that, in both groups, tolDCs induced Tregs *in vitro*. However, only the tolDCs derived from well-controlled T1D patients decreased the T helper (Th)1/Th17 responses and suppressed

the activation of antigen-specific T cells, unlike the tolDCs derived from patients with a deficient metabolic control. Additionally, the functionality of these tolDCs was evaluated in an adoptive transfer model of NOD-SCID mice, resulting in a delay in the onset of the disease[20].

The relevance of the activation state of each T1D patient in the functionality of tolDC strategies is strengthened due to the evidence obtained with human cells by our research group. Our results showed that alternatively IL-10/TGF-β1-generated tolDCs effectively induce insulin-specific tolerance in autologous effector/memory CD4+ T cells derived from T1D individuals, without affecting the proliferative response to an unrelated antigen. TolDC-stimulated T cells reproducibly displayed a decrease in activation molecules and pro-inflammatory cytokines (IL-2, IFN-γ), with high levels of the anti-inflammatory cytokine IL-10 and exhibition of an anergic state. Nevertheless, the degree of tolerance induction was dependent on the initial T cell activation state of each patient[44]. These results agreed with another study with IL-10/TGF-β-generated, insulin, or GAD65-loaded tolDCs from T1D patients, which similarly showed antigen-specific autoreactive cell hypo-responses, lower IL-2 and IFN-γ secretion, and higher IL-10 production by T cells[58]. These studies demonstrate the ability of *in vitro*-generated tolDCs to induce antigen-specific tolerance in T cells.

Liposomes or apoptotic bodies: Apoptosis is an effective mechanism to induce tolerance. The capture of apoptotic bodies by APCs (macrophages and DCs), also called efferocytosis, is due to a specific recognition and phagocytosis through phosphatidylserine (PS)[59]. In T1D, the increase in apoptotic pancreatic β-cells or defects in efferocytosis contributes to the loss of tolerance[60]. Nevertheless, it has been shown that DCs from T1D patients may acquire defective apoptotic bodies' clearance. In a child population with T1D, the tolerogenic functionality of DCs derived from monocytes was evaluated using liposomes with PS (PS-liposomes), demonstrating that the DCs of pediatric patients with T1D phagocyte PS-liposomes function in a less efficient way than the controls, which inversely correlated with the evolution of the disease. However, the tolerogenic profile in DCs was consistent after efferocytosis[61].

DCs acquire a tolerogenic phenotype and functionality after ingestion of apoptotic β-cells and prevent T1D when transferred to NOD mice, significantly decreasing its incidence and correlating positively with insulitis reduction[62]. However, the limitation of a large source of apoptotic autologous β-cells for immunotherapeutic application is the wide outlook for a biomimicry alternative consisting of PS-liposomes containing β-cell autoantigens (insulin). However, owing to the limitation of having a large source of apoptotic autologous β-cells for their immunotherapeutic application, the need arises to extend the outlook toward a biomimicry alternative consisting of PS-liposomes containing β-cell autoantigens (insulin). Liposomes that mimic apoptotic β-cells have been shown to arrest autoimmunity and prevent T1D through the generation of tolDCs. These DCs exposed to PS-liposomes decrease the proliferation of autologous T cells, deregulate genes associated with antigen presentation, and increase tolerogenic genes as well as anti-inflammatory pathways[63]. A similar study establishes that insulin-loaded PS-liposomes also reduce the severity of insulitis and that the administration of PS-free liposomes demonstrates the importance of PS in modulating the expansion of antigen-specific CD4+ T cells[64]. Immunotherapy based on the use of liposomes constitutes a promising strategy for autoimmune diseases, including T1D.

TLR ligand: Hayashi *et al*[65] proposed an innate immune response modulator generated by conjugating a TLR-7 Ligand to six subunits of polyethylene glycol, "PEGylated TLR-7 Ligand", or 1Z1. DCs treated *ex vivo* with 1Z1 and injected into NOD mice delay the appearance of insulitis, suggesting that 1Z1-treated DCs are functionally tolerogenic since these cells suppress the proliferation of antigen-specific T cells. Besides, these tolDCs do not promote an inflammatory response *in vitro* or *in vivo* and show an increase of the expression of PD-L1 and IL-1 receptor-associated kinase M.

TLR-2 involvement in T1D development has been shown by the late apoptotic β-cells ability to stimulate APCs through this receptor, contributing to activate diabetogenic T cells. Hence, as a T1D therapeutic alternative, TLR-2 blocking or tolerization is proposed. TLR-2 tolerization was carried out with a prolonged treatment of the agonist (Pam3CSK4). This treatment attenuates T cell activation mediated by DCs[66]. Furthermore, the combination of this therapy with the inhibitor (DA-1229) of dipeptidyl peptidase 4, which increases the mass of β-cells, can reverse the appearance of diabetes in NOD mice[67].

Perspective obtained from clinical trials with the use of tolDCs for T1D therapy

Clinical trials with good progress, but limitations and barriers: The first tolDC-based clinical trial for T1D treatment supports their safety administration (Clinicaltrials.gov identifier: NCT00445913)[68]. In this protocol, alternatively generated tolDCs were developed with antisense phosphorothioate-modified oligonucleotides targeting the transcripts of the costimulatory molecules CD40, CD80, and CD86. Available data show that the administration of these tolDCs upregulate the frequency of a B-lymphocyte subpopulation that was later discovered to possess immunosuppressive capability[49,51]. In this study, the procedures, equipment, and facilities comply with recommendations and are approved by the Food and Drug Administration (FDA), and no toxicity or adverse effects associated with the tolDC therapy were reported. Hence with FDA and Institutional Review Board approval, a new phase 2 study was started. This clinical trial in phase 2 (Clinicaltrials.gov identifier: NCT02354911)[69], aims to assess the capability of these tolDCs to disrupt the autoimmune process leading to β-cell destruction in individuals with T1D. To evaluate the expected effect, indirect studies will be carried out with C-peptide measurement, glycosylated hemoglobin A1c, and basal and postprandial glucose. The investigators will mainly evaluate the number of potentially tolerogenic/Tregs, B cells, and DCs and also aim to identify molecular signatures of these cell populations and correlate them with the clinical response. However, the status of this phase 2 clinical trial is unknown. It is worth mentioning that it has been reported that these tolDCs generated with antisense oligonucleotide induce T cell anergy[40].

On other hand, as have been reviewed in preclinical section, several preclinical studies have documented the ability of tolDCs loaded with antigen to induce antigen-specific tolerance. In this line, the intradermal administration of proinsulin peptide-pulsed tolDC, showed no signs of systemic immune suppression, no induction of allergy to insulin, no interference with insulin therapy, and no accelerated loss in β-cell function in patients with the remaining C-peptide level, assuming the tolDC therapy appears to be feasible and safe[70]. Yet, this study shows that the residual β-cells' function assessed by C-peptide detection did not change after tolDC administration. However, it´s important to highlight that this study was carried out with long-standing T1D patients, and this aspect get special attention owing to preclinical data point out that the efficacy of tolDC to promote optimal tolerance to specific antigen might be useful just in certain subsets of T1D patients, since the extent grade of disease (metabolic indicator as uncontrolled glycemia, uncompensated patients, activate state of T cells), being a barrier to get optimal effectiveness[20,44,51].

It is worth mentioning that the clinical trials described are carried out according to the standards for effector immune cells regulated by the foundation for the accreditation of cellular therapy[71].

Perspectives for TID therapy: Despite the existence of data showing optimal regulatory properties of tolDC, which might encourage the rising of additional clinical trial studies, important aspect must be considered; for instance, given the uncertainty of DCs' plasticity under inflammatory microenvironment prolonged, the road for the safe use of tolDC vaccines in T1D patients has been taken into account. In addition, owing to the grade of the disease may reflect distinctive efficacy, spotlighting the interest in testing patients with a shorter clinical diagnosis, where tolDC might delay the progressive autoimmune process.

In line with the aforementioned, one phase 1 study evaluating the use of tolDC as immunotherapy vaccine for the treatment of patients with T1D who use insulin and don't have any other diabetes-related health complications, is currently driven (Clinicaltrials.gov identifier: NCT04590872)[72]. Here, the safety and viability of autologous tolDCs loaded with proinsulin peptide "C19-A3" (PIpepTolDC) in new-onset T1D patients is evaluated, being C19-A3, a pharmaceutical product regulated by FDA. The PIpepTolDC vaccine aims to protect β-cells to lead an efficient insulin production to control blood glucose levels and reduce T1D-related complications by reducing the autoimmune process. The clinical effect in this study is evaluated by measuring levels of glucose, C-peptide, and hemoglobin A1c, and the effect on the autoimmune process by analyzing changes in autoantibody of pancreatic islets, T cell responsiveness, CD4^{+} T cells producing IFN-γ and IL-10, the number of autoreactive CD8^{+} T cells, as well as the immune phenotype. Thus, as perspective, the further research should encompass the viability of tolDC useful according to the clinical status of the disease.

CONCLUSION

Based on the fact that there is no specific treatment against the autoimmune process underlying T1D, which in the long-term may imply disease complications. The use of tolDC as alternative immunotherapy arises as a promising approach for T1D therapy. tolDCs have been shown to ameliorate the disease, owing to their capability to downregulate several immune cells' hyperactivity in a specific manner. Furthermore, this particular focus of tolDCs as T1D therapy is also due to the feasibility for their obtainment, since several protocols have been established, which have harnessed the DC plasticity to respond to external immunomodulatory agents, modifying in this way their phenotype, cytokine profile, and stimulatory ability, endowing them with immunoregulatory properties. Hence, various preclinical trials have demonstrated their effectiveness. Current clinical trials evaluate the safety and efficacy of tolDC administration in patients with T1D, continuing to be a viable and promising alternative therapy to reduce the autoimmune process of this disease.

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Role of insulin and insulin resistance in androgen excess disorders

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Abstract

Insulin has complex effects on cell growth, metabolism and differentiation, and these effects are mediated by a cell-surface bound receptor and eventually a cascade of intracellular signaling events. Among the several metabolic and growth-promoting effects of insulin, insulin resistance is defined as an attenuated effect of insulin on glucose metabolism, primarily the limited export of blood glucose into skeletal muscle and adipose tissue. On the other hand, not all the signaling pathways and insulin-responsive tissues are equally affected, and some effects other than the metabolic actions of insulin are overexpressed. Ovaries and the adrenal glands are two examples of tissues remaining sensitive to insulin actions where insulin may contribute to increased androgen secretion. Polycystic ovary syndrome (PCOS) is the most common form of androgen excess disorder (AED), and its pathogenesis is closely associated with insulin resistance. Patients with idiopathic hirsutism also exhibit insulin resistance, albeit lower than patients with PCOS. Although it is not as evident as in PCOS, patients with congenital adrenal hyperplasia may have insulin resistance, which may be further exacerbated with glucocorticoid overtreatment and obesity. Among patients with severe insulin resistance syndromes, irrespective of the type of disease, hyperinsulinemia promotes ovarian androgen synthesis independently of gonadotropins. It is highly debated in whom and how insulin resistance should be diagnosed and treated among patients with AEDs, including PCOS. It is not suitable to administer an insulin sensitizer relying on only some mathematical models used for estimating insulin resistance. Instead, the treatment decision should be based on the constellation of the signs, symptoms and presence of obesity; acanthosis nigricans; and some laboratory abnormalities such as impaired glucose tolerance and impaired fasting glucose.

Key Words: Insulin; Insulin resistance; Hyperinsulinemia; Hyperandrogenism; Androgen excess

Core Tip: In patients with insulin resistance, not all signaling pathways and insulin-responsive tissues are equally affected, and some effects other than the metabolic actions of insulin are overexpressed. Ovaries and the adrenal glands are two examples of tissues remaining sensitive to insulin actions where insulin may contribute to increased androgen secretion leading to androgen excess disorders. Therefore, the role and contribution of hyperinsulinemia triggered by (selective) insulin resistance has paramount importance for elucidating the pathogenesis of these disorders and establishing the right patient for insulin sensitizer therapy.
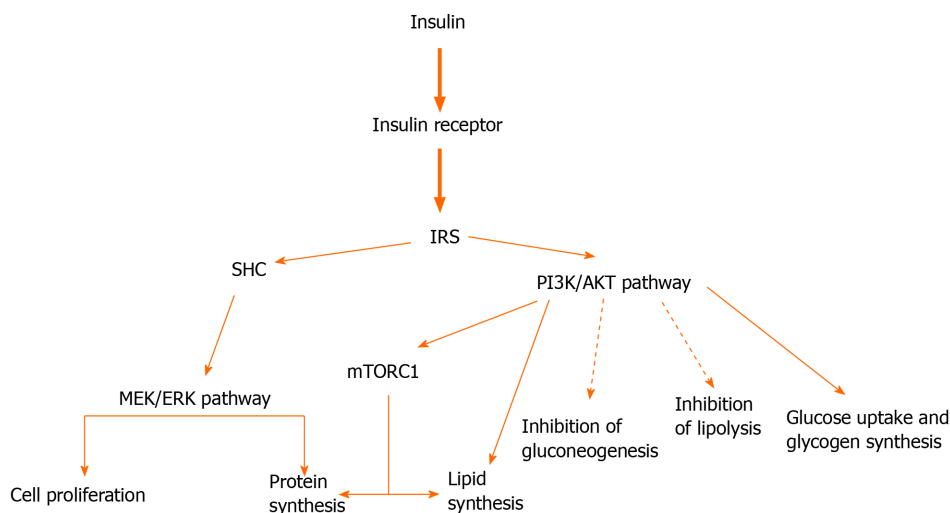
INTRODUCTION

Androgen excess disorders (AEDs) affect approximately 10% of childbearing women. Most of these patients suffer from hirsutism. Various pathogenetic factors play a role in the evolution of these disorders. Most of these disorders are associated with metabolic abnormalities during the course of the disease. Insulin resistance and hyperinsulinemia are the main contributors to metabolic derangements and are players in the pathogenesis of some of these disorders. In this review, we present the relationship among insulin resistance, hyperinsulinemia and AEDs.

INSULIN AND INSULIN SIGNALING

Insulin is an anabolic hormone and is secreted by the beta cells of the pancreas. Although it has a large number of cellular responses/effects, maintaining glucose homeostasis is considered the main physiological function of insulin. Insulin exerts its effect on muscle cells to promote glucose uptake and protein synthesis; in adipose tissue, insulin promotes fatty acid and glucose uptake and inhibits lipolysis; and in the liver, insulin suppresses glucose production. Insulin has complex effects on cell growth, metabolism and differentiation, which and these effects are mediated by a cell-surface bound receptor and eventually a cascade of intracellular signaling events[1,2] (Figure 1).

The insulin signaling cascade is composed of reversible enzymatic reactions. The cornerstone of insulin signaling is the sequential phosphorylation of downstream targets. Following insulin binding, insulin receptor tyrosine kinase is activated, leading to tyrosine phosphorylation of the insulin receptor. The autophosphorylation of the insulin receptor leads to tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and Src homology 2 domain-containing transforming proteins (SHCs)[3]. There are four isoforms of IRS; however, isoforms 1 and 2 are the main isoforms involved in metabolic actions[4]. In addition to the differences in their functions, these isoforms show different tissue distributions, thus leading to pleiotropic actions of insulin. IRS proteins are adaptor proteins that convert the tyrosine phosphorylation signal into a lipid kinase signal *via* the catalytic subunit of the enzyme phosphatidylinositol-3-kinase (PI3K). During the insulin signaling cascade, tyrosine phosphorylation is activated, while serine/threonine phosphorylation inactivates insulin receptor and IRS proteins[5]. In general, although the effects are site-dependent, the major mechanism of the termination of insulin receptor signaling is serine/threonine phosphorylation.

The phosphorylation of IRS leads to the binding of PI3K and the synthesis of phosphatidylinositol-triphosphate. These intracellular cascades lead to the phosphorylation and activation of serine/threonine-specific protein kinase B (AKT). There are three isoforms of AKT, and AKT2 is the most important isoform for glucose homeostasis[6]. Several substances interact, and *via* the PI3K/AKT pathway, anabolic

Figure 1 A brief scheme of the insulin signaling pathway under physiological conditions. After insulin binds to its own receptor, several pathways are activated/inactivated, resulting in an anabolic state of insulin. The autophosphorylation of insulin receptor tyrosine kinase is followed by tyrosine phosphorylation of insulin receptor substrate. The phosphatidylinositol-3-kinase/serine/threonine-specific protein kinase B (AKT) signaling pathway promotes glucose uptake and glycogen and lipid synthesis while inhibiting hepatic gluconeogenesis and lipolysis. Moreover, AKT kinases activate mechanistic target of rapamycin complex 1, which promotes de novo synthesis of proteins and lipids. An additional insulin signaling pathway *via* Src homology 2 domain-containing transforming proteins and the mitogen-activated protein kinase/extracellular signal-related kinase pathway promotes cell proliferation and protein synthesis. Dotted lines represent inhibition, and solid lines represent stimulation/activation. IRS: Insulin receptor substrate; SHC: Src homology 2 domain-containing transforming proteins; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-related kinase; PI3K: Phosphatidylinositol-3-kinase; AKT: Serine/threonine-specific protein kinase B; mTORC: Mechanistic target of rapamycin complex.
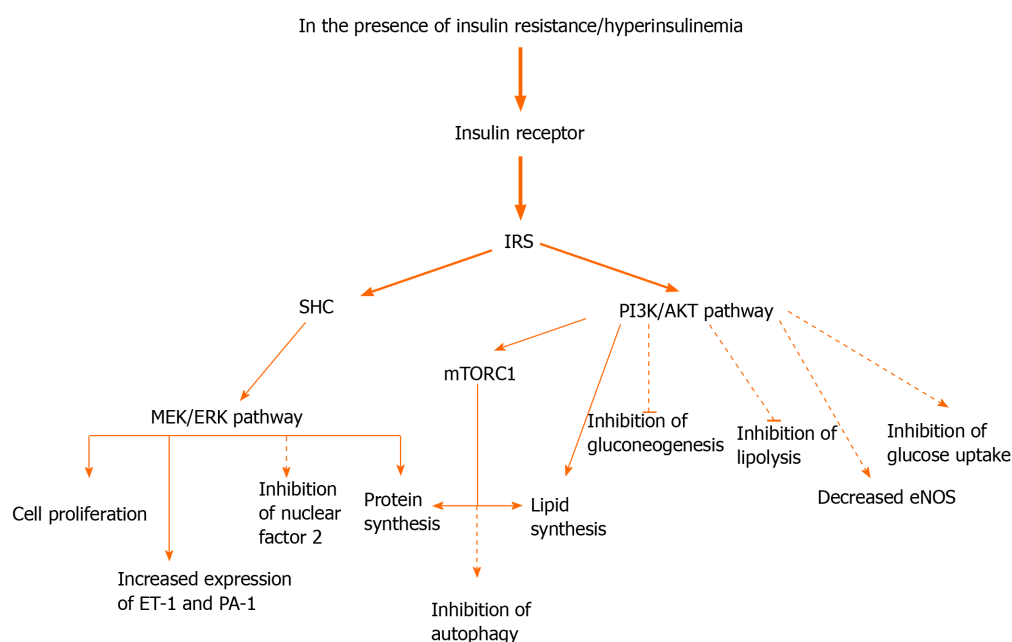
effects of insulin, such as glycogen synthesis, glucose uptake and de novo lipid synthesis, occur. This pathway also induces protein synthesis and de novo lipogenesis, which is mediated by mechanistic target of rapamycin complex 1 (mTORC1)[3].

Another insulin receptor-activated pathway is mitogen-activated protein kinase (MEK)-extracellular signal regulated kinase (ERK), which is triggered by the phosphorylation of SHC. Under physiological conditions, the activation of this pathway induces cell proliferation and protein synthesis[3,7]. There are dozens of proteins that are phosphorylated in response to insulin, and it has been shown that tyrosine phosphorylation of insulin receptor and IRS occurs within a minute upon insulin secretion/treatment[8-10]. Other downstream events occur within up to 45 min. Therefore, the occurrence of insulin receptor signaling at different times and specific patterns of phosphorylation may underlie the various responses of each insulin signaling pathway. For recent and detailed reviews on insulin signaling in normal and insulin-resistant individuals, we refer the reader to references 3 and 5.

INSULIN RESISTANCE AND HYPERINSULINEMIA

Among the several metabolic and growth-promoting effects of insulin, insulin resistance is defined as an attenuated effect of insulin on glucose metabolism, primarily the limited export of blood glucose into skeletal muscle and adipose tissue. Beta cells secrete much more insulin to compensate for and overcome insulin resistance. The resultant hyperinsulinemia is the hallmark of insulin resistance, at least at the beginning of the disease. Under physiological conditions, transient elevations in insulin concentrations are adaptive responses to environmental factors such as dietary stimuli[11]. In the case of prolonged hyperinsulinemia, there is less insulin receptor expression on the plasma membrane, which is one of the primary mechanisms of insulin resistance. However, this is not the sole mechanism of insulin resistance, and glucose homeostasis is also maintained by decreased insulin signaling *via* the PI3/AKT pathway for glucose transport from the circulation into tissues. Thus, at early stages, insulin resistance is considered a part of the defense mechanism to avoid hypoglycemia[12]. In the presence of prolonged/chronic insulin resistance and hyperinsulinemia, not all the abovementioned signaling pathways are equally affected, and relatively insulin-sensitive pathways of the insulin signaling cascade result in metabolic, vascular and reproductive dysfunctions (Figure 2).

Figure 2 A brief scheme of the insulin signaling pathway in the presence of insulin resistance. Not all insulin signaling pathways are equally affected, and selective insulin resistance is observed. (Partial) resistance in the phosphatidylinositol-3-kinase/serine/threonine-specific protein kinase B pathway results in decreased glucose uptake mediated by insufficient translocation of glucose transporter 4 and decreased inhibition of lipolysis and gluconeogenesis. Additionally, deficient activation of endothelial nitric oxide synthase is also observed. Insulin-resistance-associated hyperinsulinemia promotes anabolic cell activities *via* the mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK) pathway and *via* mechanistic target of rapamycin complex 1. In addition to the anabolic actions of signaling *via* the MEK/ERK pathway, there is also enhanced expression of plasminogen 1 and endothelin 1. The inhibition of nuclear factor 2 compromises cell defense mechanisms against radical stress. Dotted lines represent inhibition, and solid lines represent stimulation/activation. IRS: Insulin receptor substrate; SHC: Src homology 2 domain-containing transforming proteins; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-related kinase; PI3K: Phosphatidylinositol-3-kinase; AKT: Serine/threonine-specific protein kinase B; mTORC: Mechanistic target of rapamycin complex 1; GLUT4: Glucose transporter 4; ET-1: Endothelin 1; eNOS: endothelial nitric oxide synthase; PAI: Plasminogen activator.

Insulin action *via* the MAP kinase MEK/ERK pathway and partially *via* the PI3K/AKT pathway are relatively less inhibited, and these pathways promote a number of insulin-mediated functions. The activation of mTORC1 results in the suppression of autophagy, leading to a dysfunction of the turnover and removal of lipids and proteins. Insulin resistance suppresses the activation of endothelial nitric oxide synthase by AKT, and endothelial dysfunction is further enhanced by MEK/ERK-dependent expression of plasminogen activator-1 and endothelin-1. Moreover, insulin-resistance-mediated hyperinsulinemia promotes calcium influx into smooth vascular cells, leading to increased contractility and increased sodium reabsorption in renal tubules. In addition to the abovementioned systems, prolonged hyperinsulinemia also triggers functional impairments in the adrenal glands and ovaries, contributing to AEDs.

AEDs

Androgens are steroid hormones synthesized by the adrenal glands and the ovaries of women. Among the several effects on the skin, hirsutism is the main complaint of women with androgen excess. Although using some drugs, such as anabolic steroids, androgens and valproic acid, may lead to hirsutism, in most cases, the underlying cause is an AED[13]. These include polycystic ovary syndrome (PCOS), idiopathic hirsutism, nonclassic congenital adrenal hyperplasia (CAH), syndromes of severe insulin resistance and androgen-secreting tumors[14,15]. Each AED has its own pathogenesis, and different mechanisms are responsible for the increased androgen level or androgen effect. The role of insulin resistance and hyperinsulinemia in the pathogenesis of AED has been implicated for a long time. In this review, we have concentrated on the possible relationships among insulin resistance, hyperinsulinemia and AEDs; thus, the clinical manifestations and diagnostic and therapeutic aspects of AEDs are beyond the scope of this review.

PCOS AND INSULIN RESISTANCE

Theca and granulosa cell functions in normal physiology

Ovarian function is regulated by the changing levels of gonadotropic hormones (FSH/LH) as well as nonsteroidal substances such as inhibin A and B. Ovulation is the ultimate target and is the rupture and release of the dominant follicle from the ovary into the fallopian tube. The stimulation of immature oocytes by FSH results in their maturation into secondary follicles before ovulation. FSH receptors are found in the granulosa cells that surround developing ovarian follicles. Granulosa cells exclusively produce the estrogen needed to mature the developing dominant follicle. Estradiol is the main hormone of the follicle during the follicular phase of the menstrual cycle[16,17].

After the sustained elevation of estrogen levels, the characteristic midcycle LH surge causes the luteinization of granulosa cells. Prior to the LH surge, LH interacts with theca cells that are adjacent to granulosa cells in the ovary. Ovarian theca cells produce androgens that diffuse into granulosa cells and are converted to estrogen for follicular development. Luteinized granulosa cells start to respond to LH and produce progesterone. LH is responsible for inducing ovulation and induces ovarian progesterone production *via* the stimulation of theca cells and luteinized granulosa cells[16,17].

In contrast to the abovementioned physiological conditions, insulin resistance and hyperinsulinemia result in significant disturbances in ovarian functions, such as premature arrest of follicle growth and anovulation. Moreover, hyperinsulinemia has a role in amplifying LH-induced androgen production by theca cells.

Potential mechanisms of insulin resistance

In addition to being a reproductive disorder, PCOS is considered a metabolic disease associated with insulin resistance. The prevalence of insulin resistance among women with PCOS is 60%-70%; however, the detection of insulin resistance among patients with PCOS is dependent on the method used[18-20]. Although lean women with PCOS may also have insulin resistance, obesity further increases insulin resistance in those patients. Several mechanisms have been proposed for the cellular mechanisms of insulin resistance among women with PCOS. *In vitro* studies showed that the mechanisms of insulin resistance among women with PCOS are heterogeneous and involve various steps of insulin signaling. Although altered postreceptor signaling mechanisms are considered the main defect, the insulin receptor beta subunit is described as a novel molecular marker of insulin resistance. Decreased insulin receptor beta subunit has been demonstrated in several tissues, such as skeletal muscle, the liver, adipose tissue and the kidneys, during insulin-resistant states[21]. Some of the studies indicating the cellular mechanisms of insulin resistance in women with PCOS are shown in Table 1[22-24].

The role of insulin resistance and hyperinsulinemia is not limited to ovarian dysfunction. Endometrial physiology is also negatively affected since this tissue is also dependent on the action of steroids and insulin[25]. Lee *et al*[26] found that insulin receptors, IRS proteins and glucose transporters are aberrantly regulated in the endometrium of women with PCOS and are associated with hyperandrogenemia. The authors used human endometrial stromal cells (hESCs) obtained from seven healthy women and 13 women with PCOS. They demonstrated increased phosphorylation of IRS1/IRS2 on Tyr612 in androgen-treated hESCs, suggesting the role of hyperandrogenemia in the insulin signaling pathway of the endometrium. They also found that increased expression of glucose transporter (GLUT) 1 and GLUT12 was inhibited after dihydrotestosterone treatment in decidualizing hESCs. This is the only study evaluating the quantification of a series of GLUTs in the endometria of women with PCOS[26]. In addition, the insulin signaling pathway and endometrial energetic homeostasis are compromised in women with PCOS. Concomitantly, defects in GLUT4 synthesis and its translocation to the cell surface are reduced. The results obtained clearly show that molecular defects in PCOS endometria could partially explain the reproductive problems of these patients[25]. In addition to ovulatory dysfunction, blastocyst implantation and maintenance also contribute to the fertility of women with PCOS[27]. It has been shown that metformin administration to women with PCOS increases GLUT4 endometrial levels and improves the fertility of these patients.

Inflammatory cytokines are also involved in insulin resistance by triggering inhibitory phosphorylation in the insulin signaling cascade. Macrophages infiltrating adipose tissue secrete inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, which act in a paracrine manner and activate serine kinases in adipocytes[28].

Ref.	Objective	Method(s)	Main result(s)	Conclusion
Dunaif *et al*[22]	To investigate the cellular mechanisms of insulin resistance in PCOS.	Cultured skin fibroblasts from 14 women.	Increased serine phosphorylation and reduced tyrosine phosphorylation of insulin receptor.	One of the mechanisms of insulin resistance at the receptor level was demonstrated. However, 50% of women did not show this abnormality, indicating heterogeneity in the pathogenesis of insulin resistance in PCOS.
Book and Dunaif[23]	To explore the mechanisms of the paradox in metabolic and mitogenic actions of insulin.	Metabolic and mitogenic actions of insulin and IGF-1 were evaluated in cultured skin fibroblasts of 16 PCOS and 11 control women.	No difference in the number and affinity of insulin receptor in either group. Decreased glucose incorporation into glycogen in women with PCOS. Thymidine incorporation was similar between the groups.	Women with PCOS show decreased metabolic action but mitogenic action of insulin signaling was similar between the groups.
Belani *et al*[24]	To unravel insulin and steroidogenic signaling pathways in PCOS.	Insulin receptor beta subunit expression was investigated in luteinized granulosa cells obtained from 30 healthy women and 39 women with PCOS.	Compared to controls, 64% of cells show reduced insulin receptor beta subunit expression. Insulin-resistant women also showed decreased PI3 kinase expression.	Lower viability of luteinized granulosa cells in insulin-resistant women with PCOS.

Table 1 Studies demonstrating cellular mechanisms of insulin resistance in women with polycystic ovary syndrome

PCOS: Polycystic ovary syndrome; IGF-1: Insulin-like growth factor-1; PI3 kinase: Phosphatidylinositol-3-kinase.

These kinases exhibit inhibitory phosphorylation of IRS-1, thus causing insulin resistance in adipocytes. Macrophages may constitute 40% of the cells in the adipose tissue of obese individuals. Women with PCOS, particularly obese women, show an increased amount of TNF-alpha in their adipose tissue, which contributes to the development of insulin resistance[29]. It is well known that weight loss is associated with improved insulin sensitivity and metabolic parameters in addition to decreased serum and tissue TNF-alpha levels. Moreover, cytokines such as TNF-alpha may enter the systemic circulation from adipose tissue, resulting in endocrine actions and decreasing insulin sensitivity in insulin target tissues. In cultured adipocytes, TNF-alpha reduced insulin signaling by attenuating the phosphorylation of IRS proteins by insulin receptor tyrosine kinases[30].

The role of insulin in ovarian/adrenal androgen secretion

PCOS is a reproductive and metabolic disease exhibiting an insulin paradox in which ovarian and adrenal tissue remain sensitive to the stimulatory effects of insulin despite resistance to metabolic effects[31-33]. In other words, women with PCOS have a selective defect in insulin action that is characterized by resistance in metabolic signaling pathways but not in mitogenic pathways, which is particularly important in androgen production by the ovaries[33]. Patients with PCOS also have an increased adrenal androgen responsiveness to ACTH stimulation, and adrenal hyperandrogenemia is also a characteristic feature of PCOS. Some of the studies showing the effects of insulin on ovarian/adrenal hormone secretion are shown in Table 2[34-38].

Critical points in the evaluation of insulin resistance in women with PCOS

There are different phenotypes of PCOS that differ not only by the clinical spectrum of the symptoms but also by the presence/absence and the degree of insulin resistance. Euglycemic clamp studies showed that insulin sensitivity is remarkably reduced in PCOS patients who have a classic/complete phenotype, while it is less severe in those with normoandrogenic or ovulatory phenotypes[39]. It is important to note that the accurate estimation of insulin resistance in clinical studies and in outpatient clinics is a matter of debate. Although some surrogate indices of insulin resistance, such as the homeostasis model assessment (HOMA) index, have fair correlations with direct measures of insulin action, it may be misleading to categorize patients as insulin resistant or vice versa according to these parameters. There may be mismatches when using a glucose clamp and surrogate indices[19,40].

In surrogate indices such as the HOMA index, the important player is insulin, and its concentrations depend on both the metabolic clearance rate and the secretion rate from beta cells. Although there is ample evidence for the secretion of insulin, a limited number of studies have investigated the metabolic clearance rate of insulin in women with PCOS. Recently, Tosi *et al*[41] investigated the metabolic clearance rate of insulin and its relationship with the clinical, hormonal and metabolic characteristics in 190

Table 2 Studies showing the effects of insulin on ovarian and adrenal hormone secretion

Ref.	Objective	Method(s)	Main result(s)	Conclusion
Cadagan *et al*[34]	To investigate the effects of insulin and LH on PCOS theca cell CYP17 expression and androgen secretion.	Cells were obtained from three women with PCOS and three healthy women.	PCOS theca cells exhibit increased CYP17 enzyme activity/expression and increased androgen secretion.	There is a defect of steroid biosynthesis in ovarian theca cells, which is further augmented under hyperinsulinemia and increased LH secretion.
Munir *et al*[35]	To define the intracellular signaling pathways that link the insulin receptor to androgen biosynthesis.	Third-passage human ovarian theca cells were used.	Insulin regulation of 17-alpha hydroxylase activity is mediated by PI3 kinase.	Insulin stimulates ovarian androgen production, which is different from the effects on glucose metabolism.
la Marca *et al*[36]	To test the hypothesis of the linkage of hyperinsulinemia and abnormal activity of P450CYP17.	HCG test before and one month after metformin (1500 mg/d) therapy in 11 women with PCOS	After metformin, women with PCOS had significantly lower insulin and testosterone concentrations as well as lower 17-OHP responses.	Metformin leads to a reduction in stimulated ovarian P45017-alpha hydroxylase activity.
Homburg *et al*[37]	To elucidate the relationship and role of IGF-1, IGFBP-1, insulin and LH in the pathogenesis of PCOS.	Serum concentrations of IGF-1, IGFBP-1, insulin and LH in women with PCOS with or without anovulation.	Similar serum IGF-1 levels were found. However, IGFBP-1 levels were decreased in anovulatory PCOS, which is negatively correlated with insulin concentrations.	Hyperinsulinemia and raised LH are independently capable of stimulating ovarian androgen production. Growth factors may have a role in PCOS pathogenesis.
Tosi *et al*[38]	To investigate the role of hyperinsulinemia on adrenal steroidogenesis in women with PCOS.	Hyperinsulinemic clamp and saline infusion tests were performed on separate days in 12 hyperandrogenic women. Concurrent ACTH infusion to evaluate intermediate metabolites of adrenal steroid biosynthesis.	Acute insulin elevation resulted in an increased response of 17 alpha hydroxysteroid intermediates. Increased 17-OHP/androstenedione and 17-OH pregnanolone/DHEA molar ratio suggest relative inhibition of 17-20 lyase activity by insulin.	Acute hyperinsulinemia in a range found in insulin-resistant individuals enhances adrenal response to ACTH stimulation.

PCOS: Polycystic ovary syndrome; LH: Luteinizing hormone; PI3 kinase: Phosphatidylinositol-3-kinase; P450CYP17: Cytochrome 450, 17 hydroxylase; HCG: Human chorionic gonadotropin; 17-OHP: 17-hydroxyprogesterone; IGF-1: Insulin-like growth factor-1; IGFBP-1: Insulin-like growth factor-binding protein-1; ACTH: Adrenocorticotropic hormone; DHEA: Dehydroepiandrosterone.

women with PCOS. It has been shown that insulin clearance is remarkably reduced in women with PCOS compared to healthy women with similar indices[41]. Moreover, in multivariate analysis, body fat, estimates of insulin secretion and levels of serum androgens were all independent predictors of insulin clearance, and they all had negative relationships. The authors revealed that obesity contributes to hyperinsulinemia by both lowering insulin metabolism and increasing insulin secretion in addition to regulating insulin clearance by serum androgens.

Although insulin resistance is associated with PCOS, it is well known that not all women with PCOS have insulin resistance and hyperinsulinemia. Baillargeon *et al*[42] evaluated 100 nonobese women with PCOS with normal indices of insulin sensitivity indicated by normal glucose tolerance, fasting insulin, peak insulin during an OGTT and fasting glucose/insulin ratio. Those women received 850 mg metformin twice daily, 4 mg rosiglitazone, a combination of both drugs or at least one placebo for six months. In comparison to placebo, insulin sensitizers significantly improved ovulation. After treatment, serum testosterone levels also decreased significantly in comparison to the placebo group. The authors suggest that there is a subgroup of women with normal insulin sensitivity, and even those patients may benefit from insulin-sensitizing therapies in terms of the resumption of menses and improvement in hyperandrogenemia[42].

Several studies have demonstrated the role of insulin resistance in the pathogenesis of PCOS, and insulin sensitizers have been used for different clinical indications, such as metabolic effects, aiming to decrease hirsutism, resume menses and increase the ovulatory rate. If we look at the other side of the coin, do insulin sensitizers have a role in the prevention of PCOS? Ibáñez *et al*[43] investigated body composition, lipids, gonadotropins and the progression to PCOS in 24 nonobese postmenarcheal girls with hyperinsulinemic hyperandrogenemia and precocious pubarche. They were randomly assigned to receive metformin (850 mg/d) or no treatment for 12 mo. In comparison to untreated girls, metformin-treated girls had significantly improved parameters (insulin sensitivity, androgens, lipids), and the authors concluded that early metformin treatment helps to prevent the progression of precocious pubarche to PCOS. The

authors also investigated the effects of metformin (1250 mg/d) alone or in combination with an antiandrogen (flutamide, 250 mg/d) in nonobese young women with hyperinsulinemic hyperandrogenism for 9 mo[44]. In comparison to the flutamide alone group, the combination group had greater improvements in serum androgens, insulin resistance and ovulation rates (75% and 92% in the metformin alone and combination groups, respectively, but not in the flutamide alone group). These results suggest that insulin sensitizers, mainly metformin, may have a role in the early stages of PCOS and may be used as additives to other therapies, such as antiandrogens.

Apart from insulin-induced androgen secretion, androgens also contribute to the occurrence of hyperinsulinemia in women with PCOS[41]. Moghetti *et al*[45] previously assessed the effects of androgens on insulin sensitivity in 43 women (13 obese, 30 nonobese) with normal glucose tolerance and hirsutism and compared the results with those of healthy individuals matched for body mass index. Hyperandrogenic women were studied before and 3-4 mo after antiandrogen (spironolactone, flutamide, GnRH agonist buserelin) treatment. Insulin-mediated glucose uptake was lower than that in healthy individuals irrespective of ovarian or nonovarian hyperandrogenism. After antiandrogen therapy, insulin action, determined in both oxidative and nonoxidative metabolism, significantly increased, albeit it remained lower than that of the control groups. This study also showed that androgen excess per se contributes to insulin resistance and that antiandrogen therapy partially reverses peripheral insulin resistance regardless of which antiandrogen was used. These bidirectional relationships between insulin and androgens in the presence of other confounding factors are reminiscent of the relationship of the egg and the chicken.

Idiopathic hirsutism and insulin resistance

Idiopathic hirsutism is the second most common form of hirsutism and is characterized by normal serum androgen levels, normal ovulatory function and normal ovaries[46,47]. Data regarding the presence/absence of insulin resistance in patients with idiopathic hirsutism are limited in comparison to PCOS. In one of the earliest studies in this area, we investigated the presence/absence of insulin resistance in 32 patients (eight of the patients had body mass index higher than 30 kg/m^2) with idiopathic hirsutism by using basal insulin levels, HOMA scores, and OGTT and intravenous insulin tolerance test results. Patients with idiopathic hirsutism had significantly higher basal insulin levels and HOMA scores and a lower plasma glucose disappearance rate than control individuals. Six patients (18.7%) had impaired glucose tolerance (IGT); however, they were more obese than the patients with normal glucose tolerance. It is remarkable that after omitting the patients with IGT, the rest of the patients were still insulin resistant[46]. We have concluded that idiopathic hirsutism is associated with some degree of insulin resistance and an increased tendency for glucose intolerance, particularly in obese patients.

In most of the studies, lean patients with idiopathic hirsutism were also investigated to exclude the effect of obesity. Talaei *et al*[48] also investigated the presence of insulin resistance among nonobese women with PCOS (*n* = 16), idiopathic hirsutism (*n* = 30) and healthy individuals (*n* = 60). All the groups were investigated by using basal insulin levels and HOMA scores. The authors found that patients with idiopathic hirsutism had lower insulin resistance than patients with PCOS, but they had higher insulin resistance than control individuals[48]. Similarly, Sarac *et al*[49] also investigated the presence of insulin resistance among nonobese women with idiopathic hirsutism (*n* = 20) by using the euglycemic hyperinsulinemic clamp technique and compared the results with those of 20 healthy individuals. Patients with idiopathic hirsutism had lower glucose disposal rates than control individuals[49]. Although most of the studies[46,48-50] showed increased insulin resistance, opposite results have also been reported, albeit rarely. Bonakdaran *et al*[51] investigated insulin resistance in nonobese patients with PCOS (*n* = 30), idiopathic hirsutism (*n* = 30) and healthy individuals (*n* = 30) by using basal insulin levels and HOMA scores. They reported that insulin resistance was no more common than in healthy individuals. In that study, the authors classified the patients as insulin resistant (whose HOMA score > 2.68 based on a previous Iranian study) or insulin sensitive (whose HOMA score < 2.68). When they analyzed their data without classifying the patients, they again did not find any difference in insulin sensitivity between the patients with idiopathic hirsutism and healthy individuals. However, it is notable that they did not find any insulin resistance even in patients with PCOS[51].

Although it has been shown that patients with idiopathic hirsutism may exhibit some degree of insulin resistance, there are not adequate data regarding the (molecular) mechanisms of insulin resistance. Idiopathic hirsutism is considered

among AEDs; patients with idiopathic hirsutism have normal serum androgen levels, and it may be asked, how the patients exhibit similar insulin resistance as their hyperandrogenic counterparts. In that case, an important question arises: are these patients truly defined as normoandrogenic and are they truly idiopathic[52]? Previously, we showed that, although within normal limits, patients with idiopathic hirsutism have relatively higher serum androgen levels than healthy individuals[46]. In other words, those patients are actually hyperandrogenic at the tissue level; however, when we use some cutoff values derived from the reference values of commercial assays, we consider those patients to have (normoandrogenic) idiopathic hirsutism. However, those patients exhibit a lower estradiol/testosterone ratio, which is a function of aromatase activity, leading to relative hyperandrogenemia[46]. Moreover, patients with idiopathic hirsutism also demonstrate metabolic derangements compatible with insulin resistance, such as IGT[46,50].

Insulin resistance and CAH

Deficiencies in the main pathways of steroid biosynthesis lead to CAH, which is a group of disorders characterized by enzymatic defects in cortisol biosynthesis. When any mutation/mutations cause complete or near complete deficiency of the enzymes, the classic form of the disease ensues with severe clinical manifestations, such as the virilization of females or salt wasting in both sexes[53,54]. The milder form of the disease, called the nonclassic form, is typically asymptomatic at birth and is not distinguishable from other hyperandrogenic disorders, such as PCOS. Both forms of the disease differ in terms of the severity of the clinical signs and symptoms, and their treatment modalities are also different[54,55].

In patients with milder forms of CAH, glucocorticoid treatment is rarely indicated since these patients do not exhibit overt glucocorticoid deficiency. Saygili *et al*[56] investigated insulin resistance in 18 patients with untreated nonclassic CAH (NCAH), and the data were compared to those of 26 healthy individuals. Serum basal insulin levels, post glucose loading (2 h) insulin responses and HOMA scores were significantly higher in NCAH patients than in control individuals. The authors also showed a positive correlation between serum androgen and insulin levels[56]. On the other hand, glucocorticoid replacement therapy is the mainstay of therapy in the classic form of the disease, and some patients may be overtreated since the androgen suppressive dose of glucocorticoids is much more than the replacement dose. Recently, Kurnaz *et al*[57] depicted another aspect of the relationship between CAH and insulin resistance. In 56 patients with CAH and 70 healthy individuals, in addition to biochemical and hormonal investigations, the authors measured serum insulin and fetuin-A levels. Fetuin-A is a protein produced in the liver. Insulin and fetuin-A levels were significantly higher in patients with CAH than in controls, and unfavorably high levels of these proteins exhibited a positive correlation with total and free testosterone levels[57]. Since androgen receptors are also expressed in pancreatic and liver cells, high levels of testosterone can result in hyperinsulinemia. Moreover, Fetuin-A is a natural inhibitor of tyrosine kinase, and its overexpression in the liver leads to insulin resistance.

Kroese *et al*[58] investigated insulin resistance and hyperinsulinemia in 12 patients with the classic form of CAH and 12 controls matched for body mass index and age by using a euglycemic clamp. Patients were randomized to treatment with either placebo followed by pioglitazone (45 mg/d) for 16 wk or treatment with pioglitazone for 16 wk followed by placebo in a randomized crossover study design. The results of this study showed that patients with CAH who were treated with glucocorticoids were more insulin resistant than controls, and sixteen weeks of treatment with pioglitazone (45 mg/d) significantly improved insulin resistance[58].

Metformin is an oral hypoglycemic drug that has several other effects and has therefore been used in various clinical conditions[59]. Hirsc *et al*[60] investigated the effects of metformin on adrenal androgen synthesis by using human adrenal NCI-H295R cells. Cells were treated with different doses of metformin for 48 hr and tested for steroid profiles. The authors demonstrated *in vitro* that metformin reduces the activity of two important enzymes in adrenal androgen biosynthesis, 17 alpha hydroxylase/17-20 lyase and 3 beta hydroxysteroid dehydrogenase, in a dose-dependent manner by affecting the mitochondrial respiratory chain[60]. Recently, Parween *et al*[61] investigated the effect of metformin on melanocortin receptor 2 (MCR-2), which plays an important role in ACTH-mediated intracellular signaling. The authors performed the studies in an established adrenal OS3 cell model. They observed a fivefold increase in MCR-2 expression after ACTH stimulation, which was reduced 55% with metformin treatment, indicating that metformin directly affects MCR-2 expression induced by ACTH. These results provide another possible

mechanism of action by which metformin reduces adrenal steroidogenesis, and this mechanism of action may be beneficial in conditions where the hypothalamic-pituitary-adrenal axis is overactivated, such as CAH.

Although it is not as evident as in PCOS, patients with CAH may have insulin resistance, which may be further exacerbated with glucocorticoid overtreatment and obesity. Han *et al*[62] investigated whether the type and dose of glucocorticoid treatment impacts health outcomes, including insulin resistance, in 196 patients with CAH. Increasing the glucocorticoid dose with the aim of reducing serum androgen levels increased blood pressure without a remarkable benefit in disease control. The authors found that compared with those receiving prednisolone or hydrocortisone, patients on dexamethasone had lower serum androgens but greater insulin resistance. Moreover, they reported that using dexamethasone once daily was more likely to induce insulin resistance than using dexamethasone twice daily, which is explained by the higher peak of dexamethasone at night possibly inducing insulin resistance, similar to that seen in patients with primary adrenal failure[62].

On the other hand, inappropriate management of women with CAH mimics an additional PCOS-like phenotype in these women. Lifestyle modifications, changes in glucocorticoid regimens and insulin sensitizers such as metformin and/or pioglitazone may help to overcome this problem.

Syndromes of severe insulin resistance

Syndromes of severe insulin resistance are rare diseases of acquired or genetic origin. There are two main forms of these syndromes: insulin receptor gene mutations cause Type A syndrome, whereas autoantibodies against insulin receptors cause Type B syndrome. The role of insulin and insulin resistance in ovarian functions was established 45 years ago, when Kahn *et al*[63] described patients with acanthosis nigricans, hirsutism and virilization. Today, it is well known that the ovaries express not only insulin receptors but also type 1 and type 2 insulin-like growth factor (IGF) receptors, and the major hyperinsulinemia observed in Type A insulin resistance syndrome mediates its stimulatory effects *via* IGF receptors. Patients with Type A syndrome are mostly nonobese and demonstrate severe hyperinsulinemia, hyperandrogenism and acanthosis nigricans. Most of the patients are incorrectly diagnosed with PCOS. Irrespective of the type of disease, hyperinsulinemia promotes ovarian androgen synthesis independently of gonadotropins[64].

Although it is considered a subtype of PCOS, hyperandrogenism, insulin resistance, and acanthosis nigricans (HAIR-AN) syndrome is also associated with severe insulin resistance. Acanthosis nigricans is a clinical manifestation of insulin resistance characterized by velvety, hyperpigmented skin lesions mostly found on the axillary region and on the back of the neck. Abnormally increased insulin levels cross-react with insulin and IGF receptors on the ovary, leading to androgen overproduction. Metformin and/or pioglitazone may be used and have beneficial effects on serum androgen levels[65]. Recently, in five women with HAIR-AN syndrome, liraglutide improved insulin resistance, serum androgen levels and menstrual abnormalities, with one pregnancy[66].

Links between hyperandrogenism and insulin resistance. How to translate in daily practice

Apart from PCOS and syndromes of severe insulin resistance, the role and contribution of insulin resistance in the pathogenesis of AEDs are a matter of debate, although several molecular and clinical relationships have been given above. Currently, it is highly debated in whom and how insulin resistance should be diagnosed and treated among patients with AEDs, including PCOS.

It is certain that insulin resistance is a common but not universal feature of PCOS. Thus, assuming all patients are insulin resistant is not logical. In daily practice, the most important problem is the correct estimation of insulin resistance. Due to its complex and time-consuming procedures, the gold-standard "euglycemic hyperinsulinemic clamp" technique cannot be applied to all women with PCOS. Instead, some surrogate markers have been suggested for the evaluation of insulin sensitivity. However, surrogate markers such as the basal insulin, glucose/insulin ratio, HOMA index, and quantitative insulin sensitivity check index are all based on basal insulin and fasting glucose and provide similar information[67,68]. Therefore, it is not suitable to administer an insulin sensitizer relying on only some mathematical models. Instead, treatment decisions should be based on the constellation of the signs, symptoms and presence of obesity; acanthosis nigricans; and some laboratory abnormalities such as IGT and impaired fasting glucose. On the other hand, given that metformin is a very

safe drug, in clinical practice, metformin may be used for trial without some sophisticated laboratory investigations if there are no other causes of concern.

Based on several molecular and clinical studies indicating the role of insulin resistance and compensatory hyperinsulinemia in PCOS pathogenesis, many drugs/compounds, including nutraceuticals, have been tested in the treatment of PCOS with the aim of weight reduction and metabolic and reproductive outcomes. Among these, metformin, orlistat, pioglitazone, inositol, glucagon-like peptide-1 agonists, and alpha-lipoic acid were all tested. A recent guideline raised by the International PCOS Network mentioned metformin as the only insulin-sensitizing agent among women with PCOS and suggested its use for weight, hormonal and metabolic outcomes in addition to lifestyle modifications[69]. Metformin has also been suggested alone or in addition to clomiphene citrate for anovulatory infertility. Additionally, the guidelines suggest considering metformin (in addition to lifestyle modification) in adolescents with a clear diagnosis of PCOS or with symptoms of PCOS before the diagnosis is established[69]. Regarding women with lean PCOS, weight maintenance through dietary interventions and obesity avoidance should be a treatment goal. Regular physical exercise has been shown to improve insulin resistance in addition to some other beneficial effects on the symptoms of PCOS[70]. Although the number of patients is limited, Anastasiou *et al*[71] showed that lean or even underweight women with PCOS may benefit from metformin therapy for the resumption of menses and ovulation. In brief, we suggest using the same principles for the decision to treat insulin resistance in patients with AEDs.

CONCLUSION

AEDs are associated with several metabolic and reproductive consequences. Data regarding insulin resistance and its role in AEDs, except for PCOS, are scarce, and current evidence shows that insulin has receptors both on the adrenal glands and the ovaries and stimulates androgen production in several ways. Increased androgens in turn trigger insulin resistance. Furthermore, obesity contributes to established insulin resistance in patients with AEDs in many ways. Future studies are needed to establish the most appropriate time for initiating therapy and candidate patients for prescribing insulin-sensitizing agents.

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Impact of spiritual beliefs and faith-based interventions on diabetes management

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Abstract

Management of diabetes constitutes significant social and economic burdens worldwide. There is a shortage of empirical studies on the management of diabetes and the associated mental health issues through spiritual beliefs and faith-based interventions (FBIs). It is not also clear how spiritual beliefs and FBIs account for the effective management of diabetic conditions. This article discusses the impact of spiritual beliefs and FBIs in the management of diabetes, from relationship and efficacy studies that report outcomes from experimental procedures of related interventions. The majority of the relationship studies showed positive relationships, while efficacy studies showed a high efficacy of interventions in faith-based approaches. However, none of the studies clearly reported the mechanisms of change or modality of operation in a FBI that can serve as a model across culture and context. Possible mechanisms of change were discussed for further development of a standard faith-based model, and finally, suggestions for future research were also highlighted by the authors.

Key Words: Comorbid health conditions; Diabetes; Faith-based interventions; Diabetes management; Spirituality; Coping strategies

Core Tip: Studies tend to show that spiritual beliefs are linked to the acceptance and

management of diabetes conditions. Other studies show that faith-based interventions (FBIs) can be useful in diabetes management. However, there is an absence of studies showing the pathway to the positive impact of spiritual beliefs and FBIs on diabetes management. We explored the relationships and effects of spiritual beliefs and FBIs on diabetes management through literature review. Mechanisms of change and directions for further research were also discussed.

INTRODUCTION

Diabetes is among the chronic diseases that plague the victims throughout their lives. Diabetic conditions have been linked to comorbid health conditions such as blindness, kidney failure, and non-traumatic lower limb amputations[1,2]. The worldwide prevalence of the lifelong disease has continuously increased from 422 million in 2014 to 463 million people in 2019, and causes about 10% of United States annual expenditures on the adult population[1,2]. About 1.6 million children and adolescents are also living with chronic illness[1]. Diabetes is among the top 10 causes of global mortality[3,4]. Diabetes accounts for increased mortality from comorbid diseases such as cardiovascular disease, stroke, chronic kidney disease, chronic liver disease, and cancer[3]. The rising global prevalence of deaths and disability-adjusted life-years due to diabetes is estimated to be about 22.9 million. Hence, about 1 in 10 persons worldwide is suffering from one type of the three diabetes including type 1 diabetes, type 2 diabetes mellitus, and gestational diabetes mellitus[5].

Patients living with diabetes experience socio-economic challenges such as loss of a job, dependence on medical and nursing care, reduced social and family interactions and changes in lifestyle[4,6]. This is because, unlike other disease conditions, where only medication is required to manage/cure, diabetes presents more complicated physiological, psychological and social conditions that make the management very difficult[7-9]. Though, diabetes may be managed through medication and lifestyle modifications such as weight loss, diet, and exercise[10,11], there is also a high need for spiritual and psychological management[8,9].

This is because amputation and other disabilities and discomfort due to diabetes account for an array of disruptions in the patients' physical, mental, and spiritual lives[12]. The spiritual health of diabetic patients can synchronize the physical, mental and social dimensions of their lives, and is necessary for coping with and management of the disease[7]. Physical disabilities imposed by diabetes such as organ amputation tends to make the patients, mentally and spiritually disabled, exposing them to elevated stress[13]. Thus, such patients seek different approaches to cope with and adapt to life.

There have been noteworthy arguments as to whether spirituality/religiosity directly affects diabetes outcomes and well-being globally. Spirituality can be a powerful coping strategy for persons with debilitating health conditions such as diabetes[14]. Several studies indicate that increased religiosity is associated with better outcomes in clinical and general populations. Religion/spirituality generates a positive attitude towards life and life experiences, making the patient dominant against ill-fated life events including disease conditions (such as diabetes) and improving life with motivation and energy[6]. This increases the tolerance and acceptance of unchangeable situations, especially when science is unable to help a patient[15]. When disease condition becomes chronic and defiles medical interventions (as is typical of diabetes), patients and physicians tend to resort to praying and spiritual approaches. Furthermore, medical researchers have acknowledged the importance of medical procedures, as well as of traditional and complementary therapies such as prayer to treating the diseases[15]. Studies also suggest that in caring for patients, medical personnel should not underscore the patients' religious beliefs[16]. This is because, people's belief about the cause, prognosis and mortality of their disease conditions affect their responses to treatment and intervention[17].

Since diabetes is a chronic and terminal condition, which needs the mental and physical involvement of the patients for management, it is necessary to consider management approaches linked to spirituality and faith. Studies have shown that spirituality and faith-based interventions (FBIs) are viable management strategies for diabetes[18-20].Religion and spirituality are frequently engaged as coping mechanisms for diabetes and other psychologically threatening conditions and have been shown to effectively improve acceptance of diabetes and self-care behavior[18]. Another study on coping and glycemic control in couples with type 2 diabetes showed that religion and faith could help in glycemic control[21].

The importance of spiritual beliefs in therapeutic practice has been demonstrated by various professional organizations in social work, psychology, and counseling, such as the Council for Social Work Education, which added it to their central aspect of human behavior interventions[22]. However, very few articles have deeply addressed the issue of spirituality and FBIs in diabetes management.

This paper adds to the quality of information available in this area. This paper examines the impact of spiritual beliefs and FBIs in diabetes management.

SPIRITUAL BELIEFS AND DIABETES MANAGEMENT

Spiritual beliefs are invaluable in the management of diabetes and other chronic health conditions. Spirituality refers to the meaning or purpose in one's life, a search for wholeness, and a relationship with a spiritual being or reality. Spirituality involves the search for meaning and purpose through which one establishes his/her relationship with time, oneself, others, and God[23]. Individual's spiritual beliefs may be expressed through religion or religious involvement, involving participation in an organized system of beliefs, rituals, and cumulative traditions[24]. Spiritual beliefs and activities can impact the management of chronic conditions through two different pathways. First, it can assist in coping with chronic illnesses by providing support, confidence, and hope, and second, it can interfere with coping resources, especially when patients neglect self-care activities and rely on prayer and/or meditation to manage their illness[25]. Empirical evidence demonstrates the relationship between spirituality and self-management of chronic diseases like hypertension[26] and diabetes[24].

Research has shown significant relationships between spiritual and religious beliefs and practices and general diet in patients with diabetes[20]. This suggests that personal adaptations of diet and other health practices such as self-care practices are linked to spiritual beliefs. Given the importance of self-care practices such as healthy food adaptation, adequate physical activity, proper medication practices, and regular glucose monitoring[27,28], the significant link between spiritual beliefs and such self-care practices suggests that spiritual beliefs impact the choice of management strategies and can make a difference in efficacy of management.

Additionally, spirituality is an imperative resource for emotional support[29,30]. In this regard, God is perceived as central in providing strength to deal with daily challenges; God is often called upon for help in controlling diabetes; and a strong belief in God, prayer, meditation, and support from church members were all sources of support. Literature shows that humans develop an increased tendency towards spirituality and religion, especially when they experience stress or chronic illnesses[31,32]. Spirituality assists in the management of patients' health by yielding positive mental effects[32]. Spirituality has also been identified as one of the important factors that affect the quality of life, quality of care, and satisfaction of patients with diabetes[33].

Hence, intervention using spiritual beliefs for the management of diabetes conditions involves utilizing any spiritual aspect in life, such as belief in a divine being, as a control to enhance self-management[34]. Some spiritual belief-related interventions are prayer, meditation, fasting, and mindful attention. Thus in a study in Black women with type 2 diabetes, religion and spirituality were related to glycemic control[35]. Furthermore, an exploratory study on the role of spirituality in diabetes management found minimal to profound impact; all participants appeared comfortable discussing spirituality within the context of strength and hope. A study conducted to explore the relationship of religiosity and/or spirituality to the self-care of diabetes[24] showed religion or spirituality as coping methods and social support. Studies have indicated that religious involvement is associated with better adaptation to chronic diabetes by improving attendance at scheduled medical appointments, and better compliance with medication[36]. Table 1 shows the results of previous studies on spiritual beliefs and diabetes management[37-41].

Table 1 Empirical results on the impact of spiritual beliefs on diabetes management

Ref.	Study objective	Method/sample	Result
Darvyri *et al*[9]	To evaluate the impact of spirituality/religiosity on. T2DM management and to summarize the evidence regarding T2DM outcomes, as they are related to religiosity or spirituality of people with diabetes	A qualitative study (cross-sectional)	The results showed a positive relationship between religiosity/spirituality and improved T2DM management. It also suggests that participation in church and spiritual beliefs had ameliorating effects on stress levels and thus, on glycemic control of these patients with diabetes
Irajpour *et al*[29]	To explore the spiritual aspects of care for chronic Muslim patients	A qualitative-descriptive exploratory study was conducted in Isfahan, Iran, on a purposive sample of 25 participants, including patients, caregivers, nurses, physicians, psychologists, social workers, and religious counselors	The spiritual aspects of care for chronic Muslim patients fell into four main themes. Among the four major themes was the religious aspect, including doing religious rituals, attention to religious values, and providing the possibility of performing religious practices. The second theme is the pastoral aspect, which consisted of giving consultation for finding the meaning of life/death, achieving intellectual transcendence, and improving the patient's communication with herself/himself and others
Amadi *et al*[37]	To assess the association between religiosity, religious coping in depression and diabetes mellitus, and selected socio-demographic variables (age, gender and occupational status)	Cross-sectional study (simple random sampling)	Participants in this study varied in their use of religion to cope with the stress of living with diabetes mellitus or depression according to their socio-demographic profile. Younger people with depression and diabetes used religious resources and religious coping methods to the same extent
Adejumo *et al*[38]	This study aimed to relate the psychosocial effects of religion and culture with the awareness, knowledge and attitude of Nigerians regarding diabetes prevention and care	Cross-sectional study (multi-centered random sampling)	Neglecting diabetes: 42% thought that if diabetes was neglected it could lead to kidney failure, and 23% thought it could lead to heart failure. Only 0.3% thought that neglecting diabetes could result in limb amputation 49% of patients would consult a doctor if they were ill, 43% would talk to family members, and 5% to their religious leaders. There were 7% who said they would comply with religious leaders in the management of diabetes. In terms of disease prevention, 7% of the participants would value their religious leaders
Heidarzadeh *et al*[39]	To explore the spiritual growth and its dimensions in the patients with type II diabetes mellitus	A qualitative study was conducted on adult patients with a history of at least one year of type II diabetes mellitus	The data analysis led to the emergence of 237 codes, three main themes, and seven subthemes. The primary themes included a tendency to spirituality, God-centeredness, and moral growth
Watkins *et al*[40]	To investigate the relationship among spiritual and religious beliefs and practices, social support, and diabetes self-care activities in African Americans with type 2 diabetes, hypothesizing that there would be a positive association	A cross-sectional design that focused on baseline data from a larger randomized control trial in132 participants: most were women, middle-aged, obese, single, high school educated, and not employed	Significant relationships between spiritual and religious beliefs and practices and general diet. Additional significant relationships were found for social support with general diet, specific diet, and foot care
Martinez *et al*[41]	To examined client opinions about, and experiences with religious interventions in psychotherapy	A sample of 152 clients at acounselling center of a University sponsored by the Church of Jesus Christ of Latter-day Saintscompleted a survey with ratings of specific religious interventions with regards toappropriateness, helpfulness, and prevalence	Out-of-session religious interventions were considered more appropriate by clients than in-session religious interventions, but in-session interventions were rated as more helpful

T2DM: Type 2 diabetes mellitus.

FBI FOR DIABETES MANAGEMENT

Faith-based health promotion interventions and the relationship between dimensions of religion and numerous mental and physical health outcomes have been well researched[42]. An intervention is faith-based if it arises from a church's health ministry or a special interest group[43]. Four levels or features are used to identify FBIs. The first level requires the church to be used as the recruitment site for the intervention; the second level requires that the intervention be delivered at a church; the third level includes members of local churches in intervention delivery; and the fourth level includes spiritual elements in the health message of the program[44].

FBIs have consistently reported significant health outcomes such as reductions in weight, blood pressure, glycemic, and lipid levels and increases in disease-related knowledge, physical activity, and intake of fruit and vegetables. The literature identified some spirituality issues that form the pathways for the impact of FBI on

mental health on diabetic patients to include need for empowerment, courage, hope, finding meaning in suffering grieving or anxiety; patients' uncertainty about their self-efficacy in enduring the chronic illness; difficulty expressing feelings about the situation; expressing guilt, concerns, grief and/or difficulty, as well as reflecting on joys, hopes and values; concerns regarding how caregivers are coping with illness, accepting the illness and associated mortality; and feeling of abandonment by God and others[45-48]. FBIs often adopt approaches that are culturally-sensitive and behavior-oriented and aim to foster positive health outcomes through the integration of social support[49].

Within the framework of FBI, patients with such spirituality issues can be gained from referrals to spiritual care professionals, active listening, emotional support and emotional expression; sharing of self in discussion, art, music and/or prayer; acknowledging the importance of family in the patient's life; activity and exercise; humor; examination and encouragement of spiritual practices; observing sacred and divine spiritual rituals and practices such as prayer, communion, church attendance, guided visualization, relaxing, breathing[47]. FBI for diabetes prevention and management is held in faith-based organizations such as churches, synagogues, mosques, meeting houses, and other worship places. They may be organized as congregations, national networks, or as free-standing organizations.

In faith-based organizations, diabetes management programs can be carried out using different strategies such as sharing messages with members through lectures, newsletters, and announcements; providing access to information and resources on diabetes prevention and management; partnering with community coalitions that address diabetes; arranging educational activities within the organization; offering emotional and social support; organizing workshops and programs to support healthy living through nutrition and physical activity; conducting community outreach, screening, and education; providing healthy food and activities during planned events; implementing policies that support healthy behaviors within the organization.

FBI PROCEDURE

FBIs have been criticized for the absence of methodological rigor in many efficacy/effectiveness trials[50-52]. They generally utilize specific spiritual modalities such as prayers, meditation, voluntary fasting, sacred writing, focusing, journal writing and rituals[33,53-56]. Prayer as an intervention can be a vehicle for creating cognitive change[56]. The therapist can encourage clients to use prayer for coping, if appropriate, and praying in session might help to incorporate therapy into their worldview; practitioners can take advantage of clinical opportunities to use clients' prayer as a potential window into their spiritual and psychosocial functioning. Also, prayer might be used as a vehicle for creating cognitive-behavioral changes[57].

Meditation can be used as a method to attain a balanced lifestyle, and the topic of lifestyle balance can be introduced early in the clinical process. After discussion and questions about meditation are completed with the client in the session, the client should be given instructions for a practice session in the office[58]. Sacred writings, also known as religious bibliotherapy[59,60] can be used when it is determined to be of value to the client, and the particular writings can be examined at least cursively in advance by the therapist. Miller *et al*[61] notes that such materials are useful for self-help, education, psychosocial support, and interaction. Focusing technique is defined as "the vague, bodily, holistic sense of the situation such as a problem, creative project, or spiritual experience" Miller *et al*[61]. Through this intervention, the clients may learn to listen to themselves without judgment. Journal writing may be in the form of chronology, recollections and focused analysis. The intent is to help the client feel free and safe. Clients often learn to trust themselves and learn their inner thoughts and feelings and find inspiration. To effectively implement FBIs, Dodd[62]observed that it is very important to have the keenness and capability to incorporate spirituality into the psychotherapeutic process when appropriate. Lancaster *et al*[63] observed that the use of faith-based organizations can provide opportunity for the delivery of positive health messages and fostering of acceptance of healthy behavior due to the relevance of faith to many client populations. Another means of modality in FBIs is rituals. Rituals are religious or secular formalized behavior patterns that draw out certain feelings. They include creating a sacred space, the expectation of a change in insight, attitude, affect, or the receipt of guidance, and the expectation of awareness of the transcendent[64].

In a systematic review, Lancaster *et al*[63] notes that FBIs targeting changes at both the church and individual levels would have a greater impact on weight loss and related behaviors than interventions targeting a single level; interventions involving lay health advisors (LHAs) would be more successful in facilitating behavior change than investigator-led interventions. When LHAs facilitate the implementation of health programs faith-based organizations their relationships and familiarity with key church personnel, procedures and members can help facilitate outcomes[63]. The research further showed that FBIs that include religious or spiritual components (*e.g.*, scripture, biblical concepts) would lead to greater improvements in outcomes than faith-placed interventions based on surface-level characteristics (*e.g.*, race, commonly eaten foods), including conducting programs in culturally appropriate settings[63]. Hence the model of the process of FBIs is based on cultural background, spiritual perspective, and relationships, all of which are embedded in social-cognitive modalities.

IMPACT OF FBI ON DIABETES

Faith-based therapeutic interventions have been widely applied in managing diabetes and related variables across the world. An FBI on a multi-component curriculum including Scripture readings, prayer, goal-setting, a community resource guide, and walking competitions showed a decreased systolic blood pressure by 12.5 mmHg among intervention participants and only 1.5 mmHg among controls (P = 0.007)[47]. In a preliminary study[64], presented the results of "faith on the move", a randomized pilot study of a faith-based weight loss program for black women. The study's goals were to estimate the effects of a 12-wk culturally tailored, faith-based weight loss intervention on weight loss, dietary fat consumption, and physical activity in overweight/obese black women. Although the results were not statistically significant, the effect size suggests that the addition of the faith component improved results.

Sattin *et al*[65] used a "fit body and soul (FBAS)" (an FBI program) for diabetes prevention to reduce weight and fasting plasma glucose (FPG) and increase physical activity from baseline to week-12 and to month-12 among overweight parishioners and recorded a significant decline in FPG in FBAS compared to the comparison group. In a methodological review, another study[47] found that faith-based organizations may be a promising avenue for delivering diabetes self-management education to Black Americans.

Another study on faith-based diabetes prevention program (fine, fit, and fabulous) for Black and Latino congregants at churches in low-income New York City neighborhoods, which included nutrition education and fitness activities while incorporating bible-based teachings that encourage healthy lifestyles, accounted for statistically significant change in participants' dietary habits[66]. Participants reported that they ate less fast food and were less likely to overeat at follow-up. The average weight loss across churches was 4.38 pounds or 2% of participants' initial body weight. Churches and other faith-based organizations are increasingly popular settings to conduct health promotion programs[48]. Table 2 shows the works conducted so far on the impact of FBIs on diabetes management. Table 2 suggests that all the studies found a positive impact of FBI in the management of diabetes across populations[67-70].

MECHANISMS OF CHANGEFOR FBIS FOR DIABETES MANAGEMENT

Considering that FBIs are efficacious in the management of diabetes, it is right to propose that such interventions work with multi-modal mechanisms, affecting different dimensions of the illness. FBI has positive effects on the prevention, self-management and mental health of patients with diabetes[49]. This suggests that FBI may take multiple pathways to affecting different dimensions of diabetes, however, little is known about the mechanisms of change in the area of FBIs for diabetes management. Mechanisms of change explain the key processes within a therapeutic intervention that are crucial to clinical change. Investigating mechanisms of change can help to identify and preserve the ingredients of an intervention which must not be diluted to achieve change and can enable the development of more effective treatments[71].

In the case of FBI for diabetes management, some of the paramount mechanisms are increasing general and religious social support, strengthening spiritual beliefs and cognition, providing relevant information, and integrating health-religion relationship

Table 2 Studies on the impact of faith-based interventions on diabetes management

Ref.	Topic	Study objective	Sample	Intervention	Result
Duru *et al*[47]	Sisters in Motion: A randomized controlled trial of a faith-based physical activity intervention	To evaluate a faith-based intervention ("Sisters in Motion") intended to increase walking among older, sedentary African American women	Sixty-two African American women > 60 yr	Multi-component curriculum including scripture readings, prayer, goal-setting, a community resource guide, and walking competitions. Both intervention and control participants participated in physical activity sessions	At 6 mo, intervention participants had increased their weekly steps by 9883 on average, compared to an increase of 2426 for controls (*P* = 0.016); SBP decreased on average by 12.5 mmHg among intervention participants and only 1.5 mmHg among controls (*P* = 0.007)
Fitzgibbon *et al*[64]	Results of a faith-based weight loss intervention for black women	The goals of the study were to estimate the effects of a 12-wk culturally tailored, faith-based weight loss intervention on weight loss, dietary fat consumption and physical activity	Fifty-nine overweight/obese black women were randomized to one of the two interventions	"Faith on the Move," intervention	Although the results were not statistically significant, the effect size suggests that the addition of the faith component improved results.
Sattin *et al*[65]	Community trial of a faith-based lifestyle intervention to prevent diabetes among African-Americans	To reduce weight and fasting plasma glucose and increase physical activity from baseline to week-12 and to month-12 among overweight parishioners through a faith-based adaptation of the diabetes prevention program called "FBAS"	604 African Americans, aged 20 to 64 years single-blinded, cluster-randomized, community trial	FBAS is an adapted faith-based diabetes prevention program	FBASparticipants had a significant difference in adjusted weight loss compared with those in HE (2.62 kg *vs* 0.50 kg, *P* = 0.001) at 12-wk and (2.39 kg *vs* − 0.465 kg, *P* = 0.005) at 12-mo and were more likely (13%) than HE participants (3%) to achieve a 7% weight loss (*P* < 0.001) at 12-wk and a 7% weight loss (19% *vs* 8%, *P* < 0.001) at 12-mo.
Gutierrez *et al*[66]	Health, community, and spirituality: Evaluation of a multicultural faith-based diabetes prevention program	To evaluate FFF, a faith-based diabetes prevention program for black and Latino congregants at churches in low-income New York City neighborhoods	Participants (*n* = 183)	FFF, a faith-based diabetes prevention program. FFF is a 12-wk, bilingual program developed by the Bronx Health REACH Coalition, FFF includes nutrition education and fitness activities while incorporating Bible-based teachings that encourage healthy lifestyles	Participants reported statistically significant improvements in knowledge and healthy behaviors from baseline. Increased numbers of participants reported exercising in the past 30 d, eating fruit daily, being able to judge portion sizes, and reading food labels
Frank *et al*[67]	A faith-based screening/education program for diabetes, CVD, and stroke in rural African Americans	To investigate the effectiveness of a faith-based screening/education program for reducing diabetes, cardiovascular diseases, and stroke in rural African Americans	120 parishioners from African American churches	The program included education about the prevention of diabetes and cardiovascular diseases	Positive feedback was recorded by both pastors and participants
Rhodes *et al*[68]	Cost-effectiveness of a faith-based lifestyle intervention for diabetes prevention among African Americans: A within-trial analysis	To assess costs and cost-effectiveness of implementing FBAS, a church-based 18-session lifestyle education intervention for African Americans	604 overweight participants in 20 churches	FBAS, a church-based 18-session lifestyle education intervention	Per-person intervention cost of FBAS was $50.39 more than HE ($442.22 *vs* $391.83 per person), and adjusted differences in weight change (1.9 kg [95%CI: 1.0-2.8]) and waist circumference (2.4 cm [95%CI: 1.3-3.4]) were both significant. For a modest increase in cost, FBAS led to greater weight and waist reductions among African Americans in a church setting
McElfish *et al*[69]	Design of comparative effectiveness of a randomized controlled trial testing a WORD DPP *vs* a PILI DPP for Marshallese in the United States	To investigate the comparative effectiveness trial testing 2 DPP interventions designed to reduce participant's weight, lower HbA1c, encourage healthy eating and increase physical activity	384 Marshallese participants from 32 churches located in Arkansas, Kansas, Missouri, and Oklahoma	WORD DPP focuses on connecting faith and health to attain a healthy weight, eat healthily, and be more physically active. In contrast, PILI DPP is a family and community-focused DPP curriculum specifically adapted for	Ongoing

				implementation in Pacific Islander communities
Goode[70]	The effect of a diabetes self-management program for African Americans in a faith-based setting (pilot study)	To test a 6-wk faith-based diabetes self-management program for African American adults diagnosed with diabetes	32 African Americans 18 yr or older participate in the study	Diabetes self-management education intervention	There were significant improvements among participants in diabetes knowledge, self-efficacy, diabetes symptom management, and improvements in diabetes self-care activities (diet, exercise, and foot care)

CVD: Cardiovascular disease; DPP: Diabetes prevention program; FBAS: Fit body and soul; FFF: Fine, fit, and fabulous; PILI DPP: Pacific culturally adapted diabetes prevention program; WORD DPP: Faith-based diabetes prevention program.

through improving emotion regulation and cognitive restructuring[71,72]. For clarity, Figure 1 provides the pathways to changes in diabetes management due to FBI. Hence, we proposed that providing FBI for diabetes management culturally tailored and affect different dimensions that are sensitive to diabetes prevention, management and control. Within the Social Cognitive Theory Framework, FBI would improve diabetes knowledge, self-efficacy, diabetes symptoms management, and diabetes self-management outcomes. To this end, FBI focuses on the three major dimensions, including the person (diabetes knowledge, self-efficacy, symptom management) and behavior (diabetes self-management) and the environment (the church setting). In the light of these expositions, we present a framework of FBI in the context of diabetes management as shown in Figure 1.
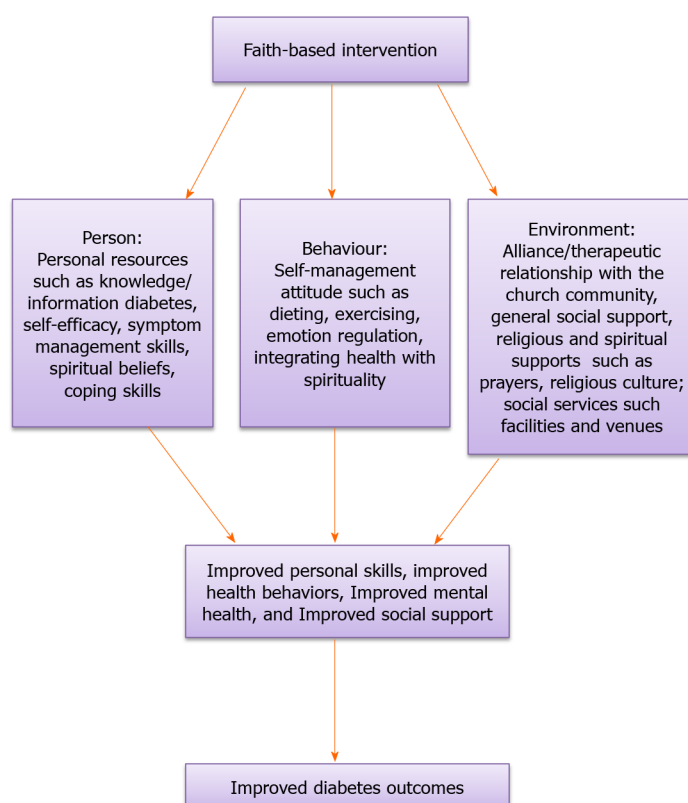
IMPLICATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

The present study has helped to illustrate the impact of FBIs and spiritual beliefs in the management of diabetes. The outcome of the study calls for emergent FBI modalities for diabetes management across the world. Further studies may attempt to develop and validate a standardized FBI program that would be useable in different religious samples. Such will provide handy, step-by-step approaches to FBI for diabetes. Researchers should attempt to increase access to diabetes management using a faith-based framework in different religious organizations. This is especially important given the place of effective management in diabetes prevention, treatment and control.

The spiritual beliefs of patients living with diabetes are of paramount impact for the purpose of maintaining good mental health of the patient[7,11-14]. Linking spirituality with health has been found to be relevant in understanding the impact of FBI in the management of diabetes[15]. Further studies are encouraged to trace the spiritual bases of diabetes management by finding out the mechanism through which spirituality affects diabetes outcomes. Given the link between spiritual variables such as prayers and beliefs and scriptures with diabetes management, and since the present study only relied on existing studies irrespective of their methodological flaws, correlation studies are encouraged, examining the impact of spiritual beliefs on diabetes outcomes. Studies should be intensified to determine the mechanisms of change in the FBI for diabetes management through experimental approaches. This will help determine the specific faith-based factors that account for positive change in diabetes management with FBIs.

CONCLUSION

There is a tendency of spiritual beliefs to be linked with the acceptance and management of diabetes conditions and FBIs can be useful in diabetes management.

Figure 1 Framework of faith-based intervention in diabetes management. The faith-based intervention acts on the three reciprocal sources of learning according to social-cognitive theory (the person, behavior and the environment). The three sources interact to produce improved skills, health behavior, mental health and social support. Finally, the improved outcomes lead to positive outcomes in diabetes management.

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COVID-19 and hyperglycemia/diabetes

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Abstract

In early studies regarding coronavirus disease 2019 (COVID-19), type 2 diabetes mellitus was considered to contribute substantially to the disease's inflammatory response. Subsequently, even hyperglycemia, regardless of insulin resistance or diabetes mellitus, was found to be additionally harmful. Recent studies have shown inflammation of the pancreatic β cells in COVID-19, even leading to new onset diabetes mellitus. We hereby summarize core literature on glycemia and COVID-19, and present implicated pathways and mechanisms.

Key Words: COVID-19; Humans; SARS-CoV-2; Risk factors; Glucose; Inflammation; Diabetes

Core Tip: The severe acute respiratory syndrome coronavirus 2 pathogen has led to the coronavirus disease 2019 (COVID-19) pandemic. This virus exerts multi-organ actions after an initial respiratory infection. In early studies regarding COVID-19, type 2 diabetes mellitus was considered to contribute substantially to the disease's inflammatory response. Hyperglycemia in COVID-19, irrespective of insulin resistance or history of diabetes, is a portent of worse prognosis. Further studies will help elucidate the link between glycemia and COVID-19.

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogen has led to the coronavirus disease 2019 (COVID-19) pandemic. This virus exerts multi-organ actions, after an initial respiratory infection[1]. Regarding COVID-19, while age and male gender are regarded as significant risk factors, accumulating evidence suggests a strong association with an impaired cardiometabolic profile in most severely ill patients[2]. Reports from Wuhan, China were the first to indicate a higher prevalence of hypertension and diabetes mellitus (DM) among patients with severe compared to non-severe illness[3]. From the beginning of the outbreak, cardiovascular disease (CVD), obesity, type 1 DM (T1DM), type 2 DM (T2DM) and possibly hypertension have seemed to be associated with the risk of suffering or dying from COVID-19[4-6].

DIABETES AS A PREDICTOR OF THE COURSE OF COVID-19

COVID-19 patients with T2DM and/or CVD are admitted more often to intensive care units (ICUs) compared to those without T2DM or CVD[7]. Older age and T2DM are both risk factors for COVID-19, but the observation that T2DM is a disease that is frequent in advanced age, slightly confounds this association[8].The risk of developing severe COVID-19 is higher in people with DM, especially if they have other co-morbidities, thus making patients with DM an at-risk population. The worse the glycemic control, the worse the severity of infection and the greater the risk of mortality[9]. In the initial studies of COVID-19, DM appeared to be 2.26 times (95% confidence interval [CI]: 1.47-3.49) more common in patients with more severe COVID-19 compared to those with less severe infection, while at the same time the presence of DM entailed an odds ratio of 2.85 (95%CI: 1.35-6.05) of intra-hospital mortality[2]. As already mentioned, these results were not always adjusted for age, which is a major confounding factor in the prevalence of DM. In Italy, one-third of patients who died of COVID-19 had DM (median age 80.5 years) and were predominantly male (70%)[2]. Compared with the prevalence of DM in the same population segment in Italy in 2018 (20.3%), the authors reported a relative risk of diabetes of 1.75 in patients who died from COVID-19[2]. It is therefore necessary to emphasize the advanced age of patients with severe COVID-19, as well as their multiple comorbidities, defining them as a population particularly at risk.

COVID-19 AND INFLAMMATION

COVID-19 is characterized by the excessive production of inflammatory factors, leading to an "in inflammatory storm" (a combination of pro-inflammatory immuno-active molecules, such as interleukins [ILs], interferons [IFNs], chemokines and tumor necrosis factors [TNFs]) in some patients[10]. Diffuse pulmonary alveolar damage, inflammatory cell infiltration with hyaline membranes, myocardial inflammation, lymphocyte infiltration in the liver, and pancreatitis are some of the major inflammatory findings during the course of the generalized COVID-19[11]. In sharp contrast to the above, the IFN type I response is impaired in these patients[12]. For patients with severe COVID-19, this so-called cytokine storm is a potentially life-threatening event[13].

In 317 patients with laboratory-confirmed COVID-19, inflammatory responses and higher levels of IL-6 were related to disease severity[11,14]. In patients with COVID-19, inflammatory markers such as C-reactive protein, D-dimers, ferritin, and IL-6 are increased; they have a direct effect on microvascular and macrovascular structures in patients with DM[15].

DIABETES, OBESITY, AND INFLAMMATORY SIMILARITIES WITH COVID-19

Although T1DM is not related to obesity, the majority of patients with T2DM are overweight or obese. Resembling the inflammatory processes of COVID-19, prolonged hyperglycemia, regardless of diabetes type, can also impact immune function, whereas compromised immunological status is linked to macrovascular complications of DM[11].

Inflammation begets increased oxidative stress that can damage proteins, lipids and DNA, systemically, as well as locally, both in the liver and in muscles, the predominant organs that regulate glucose output and glucose metabolism, increasing insulin resistance[16]. In T2DM, inflammation occurs in the pancreatic β cell (insulitis)[16]. Macrophages play a key role in β cell inflammation, along with IL-1β signaling (a core inflammatory process in the locally stressed β cell). Along with the local injury of the pancreatic cells, lipotoxicity further deteriorates pancreatic function. Free fatty acids can also induce the local production of IL-1β- and IL-1-dependent pro-inflammatory cytokines, which target the pancreatic islets. This process also increases nitric oxide production, lowers mitochondrial ATP, causing additional β cell dysfunction, along with the release of reactive oxygen species by hypoxia and endothelial damage[17]. TNF-α is linked to insulin resistance, obesity and islet inflammation, while IL-6 promotes islet cell apoptosis; both lead to T2DM. Obesity and DM (which often are described as "diabesity") favor a switch from (anti-inflammatory) M2 macrophage predominance to (pro-inflammatory) M1 macrophage predominance, further contributing to exaggerated inflammation[18]. Of note, infection with respiratory syncytial virus increases the production of IFNγ, provokes natural killer (NK) cell activation and exacerbation of inflammation in muscle and adipose tissues. Moreover, NK cell activity was found to be lower in patients with DM; glycated hemoglobin A1c (A1c) levels are associated with NK cell activity[17].

T2DM is a disease that often occurs and/or is related to obesity. Insulin resistance and related progression to overt diabetes are strongly associated with hypertrophy and hyperplasia of adipose cells[18]. According to the World Obesity Federation, obesity-related conditions seem to worsen the effects of COVID-19; indeed, the Centers for Disease Control and Prevention reported that "people with heart disease and diabetes are at higher risk of COVID-19 complications" and severe obesity (body mass index of ≥ 40) entails a higher risk for severe disease or death. As previously mentioned, COVID-19 favors an inflammatory environment that may progress to a "cytokine storm" (hypersecretion of inflammatory molecules: IL-2, IL-7, granulocyte-colony stimulating factor, IFN-γ inducible protein 10, monocyte chemo-attractant protein 1 [MCP1], macrophage inflammatory protein 1-α, and TNF-α). In an analogous fashion, obesity presents a state of low-grade inflammation, as a result of the secretion of inflammatory cytokines (TNF-α, IL-1, IL-6, IL-10), transforming growth factor-β, adipokines (leptin, resistin, adiponectin), MCP-1, C-X-C motif chemokine 5, hemostatic proteins (plasminogen activator inhibitor-1), proteins affecting blood pressure (angiotensinogen) and angiogenic molecules (vascular endothelial growth factor)[13]. Hypoxia and ischemia in adipose tissue and local endothelial damage lead to the production of reactive oxygen radicals (radical oxygen species, ROS) that affect both the microenvironment and macroenvironment of blood vessels[13].
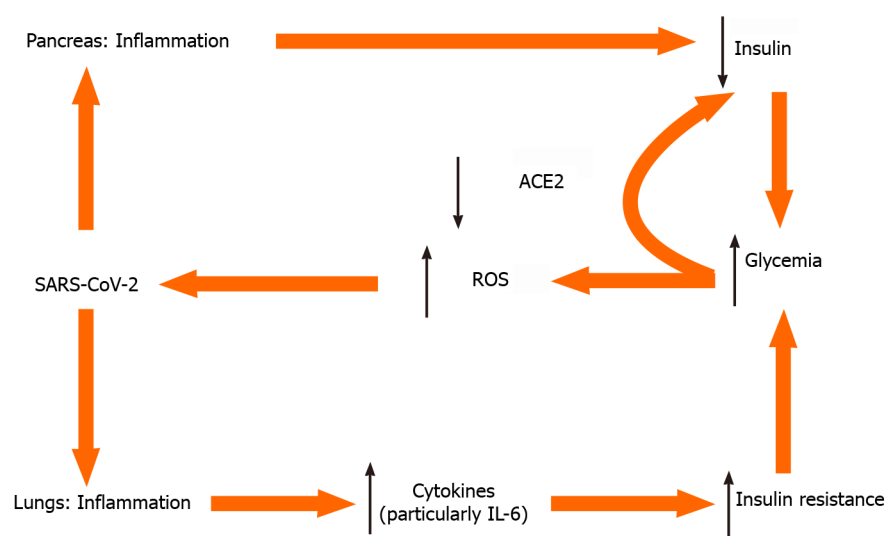
Hyperglycemia and DM affect various target organs, including the vasculature. Obesity (and its concomitant inflammation) enables another mechanism *via* which COVID-19 can provoke damage., which is directly related to the microvascular complications of DM[13].

COVID-19 AND GLUCOSE METABOLISM

Hyperglycemia was observed in patients with SARS in 2003, caused by another coronavirus, closely related to COVID-19, SARS-CoV-1) possibly due to potential transient impairment of pancreatic islet cell function. Two more *coronaviridae* ('MERS-CoV' and 'HCoV-EMC', causing Middle Eastern Respiratory Syndrome and human coronavirus;) attach to cells *via* dipeptidyl peptidase 4 (DPP-4, an enzyme that regulates insulin secretion)[19].

Glycemia on one hand is associated with SARS-CoV-2 replication[20]; elevated glucose levels and glycolysis increase SARS-CoV-2 replication and viral proliferation through the production of ROS (Figure 1). Notably, both T1DM and T2DM, are associated with a dysregulated immune response and increased morbidity and mortality[21]. On the other hand, in an inverse relationship, the presence of COVID-19 causes deterioration of glycemic control in already established DM. In a case series of critically ill, mostly not well-controlled patients with pre-existing T2DM (7 of 8 were on oral therapy before ICU admission), 85 to 480 units of insulin per day were needed to harness hyperglycemia[20].

The difference in diabetic ketoacidosis (DKA) rates in COVID-19 was four times higher in Black and two times higher in Hispanic patients with T1DM *vs* White patients with T1DM (but no statistical significance was documented)[22]. Potential

Figure 1 Selected tentative pathways for hyperglycemia in severe acute respiratory syndrome coronavirus 2 infection. ACE2: Angiotensin converting enzyme 2; IL-6: Interleukin 6; ROS: Radical oxygen species; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

explanations for these observations include the lower socioeconomic status of minority populations vis-à-vis that of the White population, the lack of appropriate nutrition and the lack of medical supervision in the use of insulin[22]. Although DKA is a major untoward event in T1DM, the majority of DKA cases with COVID-19, were observed in T2DM patients[22].

An initial diagnosis of DM was common in patients infected with SARS-COV-2, with neither any prior history of DM, nor using glucocorticoids. This new-onset hyperglycemia was an independent predictor for mortality[23] and was attributed to the binding of SARS-COV-2 to the angiotensin converting enzyme 2 (ACE2) receptor in pancreatic islets with concomitant local damage[23,24] (see also below). This ''new-onset'' hyperglycemia could be classified either as ''stress-induced'' hyperglycemia, as ''new-onset DM'' in previously unrecognized prediabetes, as hyperglycemia owing to the effects of SARS-CoV-2 to the pancreatic islets or as a result of ''secondary DM'', following use of corticosteroids[23].

Quoting the definitions of the American Diabetes Association[25], new-onset hyperglycemia without DM is defined as fasting plasma glucose (FPG) between 5.6 mmol/L and 6.9 mmol/L (100-125 mg/dL) and/or A1c between 5.7% and 6.4%, in absence of such measurements in the past. New-onset DM is defined by either of FPG > 7.0 mmol/L (> 126 mg/dL) and/or an A1c > 6.5% and/or a random glucose > of 11.1 mmol/L (200 mg/dL)[25]. Thus, abnormal glucose measurements, in the absence of A1c > 6.5% could be expected, especially during this recent viral infection (that could not have affected the A1c levels yet). Several cases of hyperglycemia or new-onset DM in COVID-19 have been reported. As might be expected, COVID-19 has been associated with severe metabolic complications of already preexisting DM, including DKA and hyperosmolarity, necessitating high doses of insulin for glycemic control.

ACE2 is expressed in the respiratory system, in the intestines, kidneys, myocardium, vasculature and pancreatic islets. SARS-CoV-2 binds to ACE2, using it as a ligand for cell entry. Interestingly, ACE2-knockout mice are more vulnerable to β cell dysfunction[24], a fact that could explain why infection with SARS-CoV-2 can cause hyperglycemia in humans without preexisting DM. After endocytosis of the virus complex, ACE2 expression is downregulated, acting in a dual way. On one hand this impairs pancreatic islet cells' function and causes β cell injury. On the other hand, downregulation of ACE2 Leads to unopposed angiotensin II action, which may further impair insulin secretion, by reducing blood flow and reducing insulin secretion while increasing oxidative stress in the pancreatic cell. Thus, coronaviruses might damage pancreatic islets, and give rise to hyperglycemia[24].

STUDIES REPORTING NEW-ONSET HYPERGLYCEMIA DUE TO COVID-19

Recently, a young 37-year-old patient with COVID-19 presented with all the clinical

features of hyperglycemia and DKA, this being possibly the first case of new-onset DM secondary to COVID-19[26]. Another case of DKA precipitated by COVID-19 in a 54-year-old patient with newly diagnosed DM was also reported[27]. Since DKA occurs as a result of insulin deficiency, such observations give rise to questions regarding the potential effect of COVID-19 in this dangerous condition[27].

In a study by Li *et al*[28], among 658 hospitalized patients with confirmed COVID-19, 42 (6.4%) out of 658 patients presented with ketosis on admission with no obvious fever or diarrhea. Patients with ketosis were younger (median age 47.0 years *vs* 58.0 years; *P* = 0.003) and had a greater prevalence of fatigue (31.0% *vs* 10.6%; *P* < 0.001), DM (35.7% *vs* 18.5%; *P* = 0.007) and digestive disorders (31.0% *vs* 12.0%; *P* < 0.001). According to their data, COVID-19 infection caused ketosis or ketoacidosis, and induced DKA for patients with DM. Ketosis increased the length of hospital stay and mortality, while DM increased the length of hospital stay for patients with ketosis but had no effect on their mortality[28].

It remains to be determined whether, after resolution of COVID-19 symptoms, glucose levels are restored to normal, thus remitting the initial diagnosis of DM. To provide answers to this conundrum, a global registry of patients with COVID-19-related diabetes has been established (COVIDIAB Project)[29].

OUTCOME IN PATIENTS WITH NEW-ONSET HYPERGLYCEMIA WITHOUT DM *VS* NORMOGLYCEMIC COVID-19 PATIENTS

Hyperglycemia (two or more blood glucose measurements > 10 mmol/L or 180 mg/dL within any 24-h period with an A1C < 6.5%), regardless of the presence of DM, is related to an increase in COVID-19 mortality compared to normoglycemia[30]. Hyperglycemia without DM is further related to increased need for mechanical ventilation, to need for ICU hospitalization and to mortality[30]. In the same gist, complications within the first month of hospital stay were increased in hyperglycemic patients without DM[31], resulting in a higher all-cause mortality[32]. Hyperglycemia at admission (but without confirmed DM) was related to a 71% increase in mortality in 1317 patients[33].
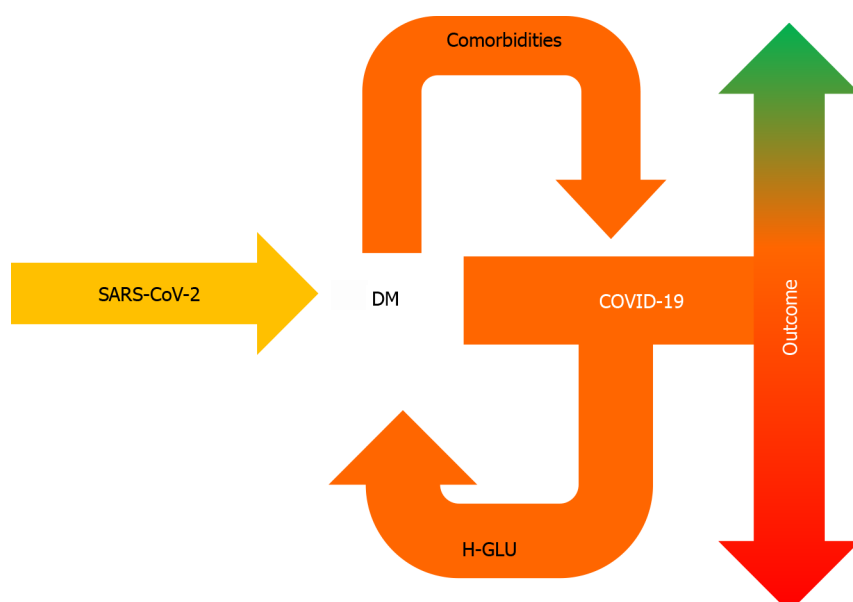
When hyperglycemia without the presence of DM was compared to known DM (new-onset and/or preexisting DM) in COVID-19 patients, a significant increase in mortality was observed among 271 patients with new-onset hyperglycemia without DM, compared to pre-existing DM. Nevertheless, ICU admission did not seem to differ significantly[34]. Critically and non-critically ill COVID-19 patients sometimes present with higher-than-expected glycemia, even in the absence of DM. Regarding the direct association of glycemia in already admitted patients in ICU due to COVID-19 infection, hyperglycemia was noted in 20 of 36 patients. Among those, none had a prior history of DM and the incidence of hyperglycemia proved to be higher that would be expected in an ICU due to stress-induced responses[35]. In a series of 157 patients with COVID-19, a substantial number of patients with and without DM presented with hyperglycemia upon admission, while critically ill patients showed compromised insulin secretion and/or impaired sensitivity to insulin[36].

OUTCOME IN PATIENTS WITH NEW-ONSET DM *VS* NORMOGLYCEMIC COVID-19

New-onset DM (and/or DKA) has been reported to occur in 16% to 21% of COVID-19 cases[26], but the incidence of complications, need for ICU and intubation, varies among studies (*n* = 413), with some showing an increase and others no difference, compared to normoglycemic patients[37,38].

OUTCOME IN PATIENTS WITH NEW-ONSET DM *VS* PRE-EXISTING DM

The risk of all-cause death in COVID-19 patients with new-onset DM is nearly double compared to that of patients with pre-existing DM[38]. A statistically significant association of ICU admission and/or of mortality in COVID-19 patients with new-onset DM (37%), compared to patients with pre-existing DM (20%) was noted; this association persisted after adjustment for age and gender[38].

Figure 2 Pre-existing diabetes mellitus can aggravate coronavirus disease 2019, following severe acute respiratory syndrome coronavirus 2 infection, whereas coronavirus disease 2019 can lead to hyperglycemia — even in the absence of diabetes mellitus, which is associated with worse prognosis. DM: Diabetes mellitus; COVID-19: Coronavirus disease 2019; H-GLU: Hyperglycemia; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Summing up the available literature, COVID-19 patients with new-onset hyperglycemia, even without a frank diagnosis of DM due to any cause (stress-induced/COVID-19-induced/pre-existing dysglycemia), show a worse course of the disease, higher rate of complications and all-cause mortality when compared to normoglycemic or patients with DM.

TREATMENT FOR COVID-19 AND GLYCEMIA

In published reports, COVID-19 patients with hyperglycemia/secondary DM were usually treated effectively with insulin[33]. In early reports, patients were also treated with hydroxychloroquine[33]. The latter medication is known to increase endogenous insulin secretion[39]. Since the use of hydroxychloroquine for SARS-CoV-2 was—at least—controversial, and has been phased out, hyperglycemia may be seen more often in patients with SARS-CoV-2 (with or without DM). Possibly higher insulin dosage—than expected—may be needed. Hyperglycemia is also to be expected by the widespread use of dexamethasone in COVID-19 patients, per the newer treatment protocols[40-46].

CONCLUSION

Hyperglycemia in COVID-19, irrespective of insulin resistance or history of DM, is a portent of worse prognosis (Figure 2). Further studies will help to elucidate the link between glycemia and COVID-19.

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Telemedicine in the COVID-19 era: Taking care of children with obesity and diabetes mellitus

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Abstract

Severe acute respiratory syndrome coronavirus 2 infection was declared a pandemic in January 2020. Since then, several measures to limit virus transmission have been imposed; among them, home confinement has been the most severe, with drastic changes in the daily routines of the general population. The "stay at home" rule has impaired healthcare service access, and patients with chronic conditions were the most exposed to the negative effects of this limitation. There is strong evidence of the worsening of obesity and diabetes mellitus in children during this period. To overcome these issues, healthcare providers have changed their clinical practice to ensure follow-up visits and medical consultation though the use of telemedicine. Telemedicine, including telephone calls, videocalls, data platforms of shared telemedicine data platforms mitigated the negative effect of pandemic restrictions. Published evidence has documented good metabolic control and weight management outcomes in centers that performed extensive telemedicine services last year during the pandemic. This review discusses studies that investigated the use of telemedicine tools for the management of pediatric obesity and diabetes.

Key Words: COVID-19; Pandemic; Children and adolescents; Obesity; Diabetes; Telemedicine

Core Tip: Quarantine confinement during the coronavirus disease 2019 pandemic has negatively impacted patient wellbeing because of difficulties in attending medical consultations. Healthcare providers have offered telemedicine support for patients with

chronic diseases, such as children with obesity and diabetes, to overcome this obstacle. Telemedicine has been shown to be effective in ensuring continuity of healthcare. Improvements are needed to reduce challenges to social inequalities in telehealth accessibility.

INTRODUCTION

In January 2019 a new severe acute respiratory syndrome (SARS) caused by a previously unknown coronavirus infection was firstly reported in China[1]. Since then, the diffusion of the so-called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or coronavirus disease 2019 (COVID-19) has reached pandemic proportion. To date, about 96 million confirmed cases and more than 2 million COVID-19 deaths have been reported worldwide by World Health Organization[2].

With the aim of containing the spread of COVID-19, several governments have imposed restrictive policies promoting social isolation and "stay at home". National lockdowns lead to sudden and radical changes in social interactions and in study and working conditions. In particular, several economic activities, school attendance, and some healthcare services have been drastically reduced and became less accessible for population. Moreover, even in nations that did not apply quarantine measures, healthcare utilization was reduced because of the fear of being infected[3]. In previous health crises, such as the SARS and Ebola epidemics, declines in healthcare utilization and delayed appointments were associated with poor health outcomes and increased mortality for chronic diseases[4,5].

Similarly, during the past year, patients with diabetes and obesity have experienced a reduced quality and continuity of healthcare support because of reduced contact with healthcare providers. To help minimize this phenomenon and adverse health outcomes, telehealth services have been rapidly implemented in several countries as an alternative to in-person visits[6]. Telemedicine is a powerful tool that allows the continuity of healthcare services; and at the same time, minimizes virus diffusion. Specific infrastructures, healthcare provider skills, and patient Internet/mobile devices are needed to allow the use of telehealth appointments (Table 1). This technology appears to be particularly helpful for the management of chronic diseases such as obesity and diabetes in the context of long-term quarantine measures.

In our Pediatric Department at the University of Campania Luigi Vanvitelli, telemedicine has been performed to ensure continuing clinical management of children and adolescents with type 1 diabetes during two lockdown periods (March-May 2020 and October-December 2020). In our experience, patients were satisfied with the telemedicine service as it allowed them to attend visits while avoiding the risk of being infected. Our patients were confident with continuous glucose monitoring (CGM) and cloud storage of records, but complained of the lack of face-to-face meeting and some Internet-connection issues. Our aim is to review the current literature about the success in applying telemedicine healthcare services in support of pediatric obesity and diabetes.

OBESITY

Indirect consequences of the COVID-19 pandemic on obesity are three-fold; increased incidence of new cases, worsening of disease severity, and barriers to access of healthcare services. In fact, quarantine measures have significantly changed children's daily routines to those favoring an unhealthy lifestyle. Stay at home restrictions have been associated with increased snacking and assumption of high-calorie foods and sugar sweetened beverages[7-9]. In addition, sports activities and outdoor play have been reduced and screen time has increased, favoring sedentary behavior[9,10].

Table 1 Telemedicine infrastructure

Privacy	Security of data should be obtained *via* platforms ensuring data encryption, integrity, and confidentiality
Technology	Instruments required to ensure good performance of telehealth visits, include Internet stability, webcams, multiaccess platforms, and access to electronic health records
Consent	Patient should give declared consent to telehealth
Billing	Each visit must be well-documented to receive reimbursement. A platform that properly documents care will help with reimbursement procedures

Longitudinal projection models have estimated an increase of pediatric obesity prevalence that is directly related to the duration of lockdown measures. United States school closures for 6 mo in 2020 has been estimated to be responsible of an increase of 2.4 percent points in childhood obesity prevalence and 0.2 *z*-score body mass index points in 2021[11]. Therefore, there is an urgent need to implement and regulate healthcare support during the COVID-19 pandemic. It should also be taken into account that medical weight management intervention for obese children and adolescents is effective when highly intensive (*i.e.* at least 26 contact h over 6 mo)[12].

To overcome these challenges, telehealth and mobile health technologies have been used alongside standard therapy for pediatric weight management. Available evidence highlights the efficacy of these tools in improving weight loss, behavioral change, and drop-out rate[13-16]. However, data about the use of health technologies as an exclusive intervention for obesity management are scarce. In New York City, the tertiary center for pediatric weight management of New York-Presbyterian, Columbia, and Weill Cornell hospitals, have exclusively performed telemedicine appointments since March 2020. The group provided nutritional, physical activity, and psychological support thanks to a virtual interdisciplinary team. The authors describe how available facilities such as shared electronic health records and the possibility of simultaneous access of several health providers, made a rapid transition to telemedicine possible. They reported an increased rate of access to telemedicine pediatric weight management visits to 76%-89% from 55%-65% before COVID[17]. This increase might be explained by the wide availability of mobile technology in the population and the reduction of barriers linked to the travel needed to reach healthcare services. However, telemedicine has concerns that should be addressed. Firstly, privacy and Internet security should be empowered using safe dedicated platforms for weight management visits that meet regulation requirements. Encryption should be provided to ensure data integrity and confidentiality. In addition, appointment billing forms should clearly describe the time spent by each healthcare provider with the patient and which platform was used for the visit. Moreover, and of great importance, assessment of weight and other parameters, such as blood pressure and heart rate, are important concerns, considering that medical decisions are made on the basis of those parameters[18]. In addition, some patients said that they were not confident with video-call appointments and preferred to wait until in-person visits will be available. That was probably due to social and economic inequalities that constitute barriers to wider acceptance of e-technologies in the population[19]. Those inequalities can be found in other countries. Some countries that already had a telemedicine infrastructure quickly implemented not-in-person healthcare. Others needed a longer time to develop efficient platforms and telemedicine assistance, which had a negative impact on patient wellbeing[20]. Long-term studies will document the effectiveness, accessibility, and feasibility of telehealth in pediatric obesity care. If found to be effective and accessible it may have potential as a key tool even in non-pandemic management.

DIABETES MELLITUS

As with obesity, home confinement imposed by governments during the COVID-19 pandemic has negatively impacted diabetes mellitus management. Limited access to physical activity, fresh foods, and healthcare services are all responsible for poor glycemic control[21]. In addition, the negative psychological effects of confinement and disruption of daily routines on therapy adherence should be considered. Previous studies reported that telehealth, including email, phone calls, and videos, were effective tools in lowering hemoglobin A1c (HbA1c) levels both in type 1 and type 2 diabetes mellitus in addition to face-to-face visits[21-24].

Several studies have evaluated the effects of changes in healthcare services during the COVID-19 pandemic on children with diabetes from the points of view of both patients and healthcare providers. An electronic survey conducted by the International Society for Pediatric and Adolescent Diabetes from March 2020 to April 2020 investigated the most common challenges and management strategies of healthcare professionals taking care of pediatric patients with diabetes[25]. The survey was completed by 215 diabetes centers in 75 countries. Most centers were in the United Kingdom and the United States. In-person visits continued in only 16.5% of the centers, and the majority of patients was followed by telemedicine, with good compliance. However, disparities in telehealth availability were reported[25]. The survey also revealed an increased incidence of severe acute diabetic ketoacidosis (DKA) as for the first time in type 1 diabetes patients. It might be hypothesized that fear of contagion delayed contact with health services, leading to more severe cases that required emergency department care[25]. Similar findings have been reported by the Italian Society for Pediatric Endocrinology and Diabetes, which registered a lower rate of newly diagnosed type 1 diabetes but a higher rate of severe DKA in 2020 compared with 2019[26].

In parallel with the lower rate of new cases, several studies conducted during the COVID-19 pandemic reported an improvement of metabolic control in this period[27-31]. Preschool and school-age children with CGM achieved better metabolic control during home confinement. The results might be explained by closer parental control of meal preparation and glucose monitoring, with consequent therapy modulation that was made possible by the "stay at home" rule[27]. In Italy, a Web-based survey revealed that the methods of communication with healthcare professionals were emails, phone calls and text messages. No cases of DKA were reported during lockdown periods. Younger patients, those who were < 12 years of age, were reported to suffer from quarantine restrictions, especially in their approach to the disease. Those patients were all monitored by remote control of CGM[29]. These technologies are a powerful tool for diabetes management, and a large percentage of patients are confident with CGM and insulin pumps. Data from those instruments can be accessed by healthcare providers who can modulate pharmacotherapy and patient compliance with treatment. Therefore, these tools form the basis for rapid application of telemedicine for diabetes care[32].

A pilot study conducted in Singapore reported good satisfaction from parents and adolescents who received telehealth monitoring of type 1 and type 2 diabetes. The patients were referred to diabetes centers only for blood collection and HbA1c monitoring. Education, therapy changes and blood test results were communicated by telehealth devices. Eighty percent of the interviewed adolescents and their parents reported no difference between face-to-face and telehealth visits, the remaining 20% was more satisfied with telehealth facilities than they were with in-person appointments[33].

Other technologies might be helpful in the telehealth management of diabetes. Remote glycemic monitoring with cloud platforms enables clinicians to continuously monitor glycemic control and to adjust therapy. In addition, multidisciplinary management including dietitians, psychologists, and diabetologists should be performed with multiaccess platforms[34]. Experience with the use of customized nutritional and physical activity counselling during quarantine, preparation of weekly food plans and in-home exercise has been reported to result in improved glycemic control[28].

However, healthcare providers have reported several concerns about exclusive use of telemedicine management for pediatric diabetes. A survey conducted in nine countries reported that most centers had insufficient technological support for teleconference appointments, which made telemedicine time consuming. In addition, instability of Internet connections impaired visit performance by increasing the duration and interfering with interpersonal relationships. Moreover, some patients in low-income countries lacked Internet connections, making telemedicine and videocalls impractical. Furthermore, there were difficulties in teaching families about data sharing and receiving information from telemedicine platforms. Another pitfall, which was highlighted by clinicians, was the impossibility of performing clinical examinations, blood tests and comorbidity screening[35]. Regarding comorbidity screening, some studies have described the usefulness of smartphone imaging devices for remote management of diabetic retinopathy. Those devices acquire non-mydriatic eye images that have been shown to have a good reliability for retinopathy screening. To date, most findings have been in adults, and no evidence is from studies conducted during the COVID-19 pandemic. However, teleophthalmology might have promise as a tool for diabetic retinopathy screening and follow-up[36].

Telemedicine appears to be a concrete approach for ensuring continuity of healthcare services for pediatric diabetes. It overcomes distance barriers and is cost effective. However, new-onset cases need in-person healthcare assistance to ensure an adequate clinical evaluation of disease severity and family education in glucose monitoring and therapy.

CONCLUSION

The COVID-19 pandemic has dramatically changed the daily life routines of patients and healthcare professionals. Clinicians had to rapidly adjust their practice to face the challenges of home confinement, and patients with chronic diseases are those who are most exposed to the negative effects of healthcare support disruption. Telemedicine is a powerful tool to address these issues, as it does not expose patients to contagion, it overcome distance barriers, and it allows a multidisciplinary team approach. However, inequalities resulting from the spread of technology infrastructures and mobile/Internet availability for some patients. Efforts to improve those facilities for both healthcare system and patients are needed.

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Basic Study

Diabetes-related intestinal region-specific thickening of ganglionic basement membrane and regionally decreased matrix metalloproteinase 9 expression in myenteric ganglia

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Abstract

BACKGROUND
The importance of the neuronal microenvironment has been recently highlighted in gut region-specific diabetic enteric neuropathy. Regionally distinct thickening of endothelial basement membrane (BM) of intestinal capillaries supplying the myenteric ganglia coincide with neuronal damage in different intestinal segments. Accelerated synthesis of matrix molecules and reduced degradation of matrix components may also contribute to the imbalance of extracellular matrix dynamics resulting in BM thickening. Among the matrix degrading proteinases, matrix metalloproteinase 9 (MMP9) and its tissue inhibitor (TIMP1) are essential in regulating extracellular matrix remodelling.

AIM
To evaluate the intestinal segment-specific effects of diabetes and insulin replacement on ganglionic BM thickness, MMP9 and TIMP1 expression.

METHODS
Ten weeks after the onset of hyperglycaemia gut segments were taken from the duodenum and ileum of streptozotocin-induced diabetic, insulin-treated diabetic and sex- and age-matched control rats. The thickness of BM surrounding myenteric ganglia was measured by electron microscopic morphometry. Whole-

mount preparations of myenteric plexus were prepared from the different gut regions for MMP9/TIMP1 double-labelling fluorescent immunohistochemistry. Post-embedding immunogold electron microscopy was applied on ultrathin sections to evaluate the MMP9 and TIMP1 expression in myenteric ganglia and their microenvironment from different gut segments and conditions. The MMP9 and TIMP1 messenger ribonucleic acid (mRNA) level was measured by quantitative polymerase chain reaction.

RESULTS
Ten weeks after the onset of hyperglycaemia, the ganglionic BM was significantly thickened in the diabetic ileum, while it remained intact in the duodenum. The immediate insulin treatment prevented the diabetes-related thickening of the BM surrounding the ileal myenteric ganglia. Quantification of particle density showed an increasing tendency for MMP9 and a decreasing tendency for TIMP1 from the proximal to the distal small intestine under control conditions. In the diabetic ileum, the number of MMP9-indicating gold particles decreased in myenteric ganglia, endothelial cells of capillaries and intestinal smooth muscle cells, however, it remained unchanged in all duodenal compartments. The MMP9/TIMP1 ratio was also decreased in ileal ganglia only. However, a marked segment-specific induction was revealed in MMP9 and TIMP1 at the mRNA levels.

CONCLUSION
These findings support that the regional decrease in MMP9 expression in myenteric ganglia and their microenvironment may contribute to extracellular matrix accumulation, resulting in a region-specific thickening of ganglionic BM.

Key Words: Type 1 diabetes; Diabetic enteric neuropathy; Neuronal microenvironment; Basement membrane; Matrix metalloproteinase 9; Tissue inhibitor of metalloproteinase 1

Core Tip: These findings demonstrate an intestinal segment-specific thickening of basement membrane (BM) surrounding myenteric ganglia. In diabetes, ganglionic BM is thickened in the ileum, but not in the duodenum. Insulin prevented the diabetes-related BM thickening. The matrix degrading matrix metalloproteinase 9 (MMP9) expression was decreased in myenteric ganglia and its environment in the diabetic ileum, however, it remained unchanged in the duodenum. Similarly, MMP9/Tissue inhibitor of metalloproteinase 1 (TIMP1) ratio decreased only in ileal myenteric ganglia. Intestinal segment-specific induction of MMP9 and TIMP1 messenger ribonucleic acid levels was revealed. Regionally decreased MMP9 expression in ganglia correlates well with segment-specific thickening of ganglionic BM and these coincide with region-dependent enteric neuronal damage.

INTRODUCTION

In the last few years, there has been an increasing emphasis on the importance of the neural microenvironment in the diabetic damage of the enteric nervous system[1-3]. Moreover, the region-specific susceptibility of enteric neurons to diabetic neuropathy and their sensibility to immediate insulin treatment[4,5] serve as further motivation to thoroughly investigate the molecular neural milieu in different intestinal segments.

Since enteric ganglia are not vascularized, the capillaries in close vicinity have a critical role to supply them with oxygen and nutrients[6]. It is well established that among others, the thickened endothelial basement membrane (BM) is one of the earliest histological hallmarks of diabetic microangiopathy contributing to impaired permeability function in retina or renal glomeruli[7-11]. Consistent with this, we revealed a strictly intestinal region-dependent thickening and separation of capillary BM in type 1 diabetic rat model[12]. Structural and morphological findings (*e.g.*, opened endothelial tight junctions, enlarged caveolar compartments, impaired distribution of endogenous albumin) suggesting altered permeability of intestinal capillaries in the vicinity of myenteric ganglia were also revealed[12].

There may be several reasons for capillary BM thickening. On the one hand, the diabetes-related enhanced expression of the prevalent BM components leads to thickened BM. High glucose-induced overexpression of collagen IV, fibronectin, laminin, agrin and tenascin was observed in different models[10,13-16]. On the other hand, due to the long-lasting hyperglycaemic condition, the decreased degradation of these BM components may also result in a thickened BM, even under good glycaemic control. In rats with diabetic nephropathy, the decreased metalloproteinase activity promotes the accumulation of collagen IV in the matrix[17]. Others also observed an accelerated matrix accumulation in metalloproteinase knockout mice[18]. Overall, the accumulation of extracellular matrix (ECM) molecules and/or the decreased degradation of matrix components may contribute to the imbalance in ECM dynamics and lead to BM thickening.

ECM proteins are degraded by several proteinases[19]. Among them, metalloproteinases are the most essential in regulating ECM remodelling[19]. Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing endopeptidases[20]. The MMP family has more than 20 members in vertebrates, most of them with basic three-domain structures and different ECM and other targets[21]. However, MMPs have not only proteinase activity, but are also implicated in other essential functions, like releasing apoptotic ligands, cytokine inactivation, cell proliferation and differentiation, angiogenesis, and host defense[19,21-23].

MMP9 is secreted by a wide variety of cells, such as macrophages, smooth muscle cells, endothelial cells, and it is one of the most extensively studied enzymes involved in ECM breakdown and turnover. Due to the crosstalk between ECM and inflammatory processes, MMPs are associated with the development of diabetic microvascular complications in different organs[24]. MMP activation accelerates apoptotic processes in retina[25]. There is correlation also between upregulated MMP expression and the progression of diabetic nephropathy[26,27]. Under diabetic conditions, MMP expression is influenced by high glucose level and reactive oxygen species[27]. Moreover, the endogenous tissue inhibitors of metalloproteinases (TIMPs), are crucial to determine the optimal proteolytic activity of MMPs[28]. Among the four members of TIMP family, TIMP1 has the strongest efficiency to inhibit most of the MMPs[29]. TIMPs can directly restrict MMP-dependent matrix proteolysis or indirectly facilitate ECM accumu-lation[29].

All enteric ganglia are surrounded with a continuous BM[6,30], and the components of this ECM sheet are not detectable inside the enteric ganglia[31]. Several studies dealt with the composition and alterations of ECM in the intestinal wall due to its impact on enteric ganglion formation during development[32-34]. The appropriate matrix composi-tion is indispensable for the development of enteric ganglia and normal nerve fibre function[33,35]. The relevance of different BM abnormalities was investigated in Hirschprung's disease[36-38], however, little is known about its role in other pathological processes, like diabetes-related enteric neuropathy.

We assume that structural and molecular alterations involved in maintaining the dynamic structure of ECM in the intestinal wall may contribute to the gut region-dependent diabetic neuropathy. Therefore, the primary goal of this study was to evaluate the effects of type 1 diabetes and immediate insulin replacement on BM thickness surrounding myenteric ganglia in different gut segments. Furthermore, we aimed to investigate the intestinal region-dependent expression of MMP9 and TIMP1 in myenteric ganglia and their microenvironment in control, diabetic and insulin-treated diabetic rats.

MATERIALS AND METHODS

Animal model

Adult male Wistar rats (Crl: WI BR; Toxi-Coop Zrt.) kept on standard laboratory chow

(Farmer-Mix Kft., Hungary) and with free access to drinking water, were used throughout the experiments. The rats, weighing 200-300 g, were divided randomly into three groups: streptozotocin (STZ)-induced diabetics (*n* = 4), STZ-induced diabetics with insulin treatment (*n* = 4) and sex- and age-matched controls (*n* = 4). The controls were treated with vehicle, while hyperglycaemia was induced by a single intraperitoneal injection of STZ (Sigma-Aldrich, Hungary) at 60 mg/kg as described previously[4,12]. The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mmol/L. From this time on, the insulin-treated hyperglycaemic group received a subcutaneous injection of insulin (Humulin M3; Eli Lilly Nederland, Netherlands) each morning (2 IU) and afternoon (2 IU). Equivalent volumes of saline were given subcutaneously to the rats in the diabetic and the control group. The blood glucose level and weight of each animal were measured weekly during the 10-wk experimental period. Those STZ-induced diabetic animals which recover spontaneously or their blood glucose level decreased under 18 mmol/L during the 10-wk experimental period did not participate in this study.

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 wk prior to experimentation.

In all procedures involving experimental animals, the principles of the National Institutes of Health (Bethesda, MD, United States) guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes were strictly followed, and all the experiments were approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority), with the license number XX./1636/2019.

Tissue handling
Ten weeks after the onset of hyperglycaemia, the animals were euthanized by barbiturate overdose (150 mg/kg pentobarbital sodium i.v. injection) for tissue collection. The gut segments of the control, STZ-induced diabetic, and insulin-treated diabetic rats were dissected and rinsed in 0.05 mol/L phosphate buffer (PB; pH 7.4). Samples were taken from the duodenum (1 cm distal to the pylorus) and the ileum (1 cm proximal to the ileo–caecal junction), and processed for fluorescent immunohisto-chemistry, quantitative electron microscopy and quantitative polimerase chain reaction (qPCR). For double-labelling fluorescent immunohistochemistry, samples (2-3 mm) from different gut segments were fixed in 4% paraformaldehyde (PFA) and embedded in melted paraffin. For electron microscopic studies, small pieces (2-3 mm) of the gut segments were fixed in 2% PFA and 2% glutaraldehyde solution and then further fixed for 1 h in 1% OsO_4. After rinsing in buffer and dehydrating in increasing ethanol concentrations and acetone, they were embedded in Embed812 (Electron Microscopy Sciences, United States). The Embed blocks were used to prepare ultrathin (70 nm) sections, which were mounted on nickel grids and processed for morpho-metrical study and immunogold labelling. For qPCR study, the 3-cm-long gut segments were cut along the mesentery and pinched flat. The layer of mucosa and submucosa was removed, and the residual material (myenteric plexus and intestinal smooth muscle layers) was snap-frozen in liquid nitrogen and stored at -80 °C until use.

Double-labelling fluorescent immunohistochemistry
For double-labelling immunohistochemistry, paraffin-sections (3.5 µm) derived from different gut segments were immunostained with MMP9 and TIMP1. Briefly, after blocking in TRIS-buffered saline (TBS) containing 1% bovine serum albumin and 10% normal goat serum, the sections were incubated overnight with anti-MMP9 (mouse monoclonal immunoglobulin G (IgG); Abcam, UK; final dilution 1:100) and anti-TIMP1 (rabbit polyclonal IgG; Santa Cruz Biotechnology, United States; final dilution 1:50) primary antibodies at 4 °C. After washing in TBS with 0.025% Triton X-100, sections were incubated with anti-mouse Cy™3 (Jackson ImmunoResearch Laboratories, Inc., United States; final dilution 1:200) and anti-rabbit Alexa Fluor 488 (Life Technologies Corporation, Molecular Probes, Inc., United States; final dilution 1:200) secondary antibodies for 1 h at room temperature. Negative controls were performed by omitting the primary antibody when no immunoreactivity was observed. Sections were mounted on slides in Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Hungary), observed and photographed with a Zeiss Imager Z.2 fluorescent microscope equipped with an Axiocam 506 mono camera.

Transmission electron microscopy

Morphometric study: For ultrathin sectioning, four Embed blocks were used for each intestinal segment and each condition (control, STZ-induced diabetics, and insulin-treated diabetics). Three grids per block were counterstained with uranyl acetate (Merck, Germany) and lead citrate (Merck, Germany) and were examined and photographed with a JEOL JEM 1400 transmission electron microscope. Montage photographs of twelve myenteric ganglia per intestinal segment per condition were made at a magnification of 20000 × and the thickness of the BM were measured at random points around the ganglia with the help of a limited size (700 nm × 700 nm) grid net. The mean thickness was then calculated for each ganglion by using the AnalySIS 3.2 program (Soft Imaging System GmbH, Germany).

Post-embedding immunohistochemistry

The Embed blocks used previously for the electron microscopic morphometry also served for the MMP9 and TIMP1 post-embedding immunohistochemistry. Ultrathin sections from each block were sequentially incubated overnight in anti-MMP9 mouse monoclonal IgG (Abcam, United Kingdom; final dilution 1:50) or anti-TIMP1 rabbit polyclonal IgG (Santa Cruz Biotechnology, United States; final dilution 1:50) primary antibodies, followed by colloidal gold conjugated anti-mouse IgG (conjugated to 18 nm colloidal gold; Jackson Immunoresearch, United States; final dilution 1:20) or anti-rabbit IgG (conjugated to 18 nm colloidal gold; Jackson Immunoresearch, United States; final dilution 1:20) secondary antibodies for 3 h, with extensive TBS washing in-between. The specificity of the immunoreaction was assessed in all cases by omitting the primary antibodies in the labelling protocol and incubating the sections only in the gold conjugated secondary antibodies. Sections were counterstained with uranyl acetate (Merck, Germany) and lead citrate (Merck, Germany) and were examined and photographed with a JEOL JEM 1400 transmission electron microscope. The quantitative properties of gold particles coding for MMP9 or TIMP1 were determined in the myenteric ganglia, the endothelium of capillaries in the vicinity of these ganglia and the intestinal smooth muscle cells. Counting was performed on digital photographs at a magnification of 20000 × with the AnalySIS 3.2 program (Soft Imaging System GmbH, Germany). Montage pictures of twelve ganglia, the entire endothelial profile of eleven well-oriented capillaries and eleven field of view of the surrounding smooth muscle cells per intestinal segment per condition were used. The intensity of the labelling was expressed as the total number of gold particles per unit area.

RNA preparation, reverse transcription and qPCR

Intestinal tissue samples were homogenized in RNA Bee reagent (Tel-Test Inc., United States) and total RNA was prepared according to the procedure suggested by the manufacturer. For assessing RNA concentration and purity, the absorbance of RNA samples was measured at 260 and 280 nm using NanoDrop 1000 UV/VIS Spectrophotometer (Thermo Scientific, United States). The RNA concentration was calculated using the A260 = 1.0 equivalent to approximately 40 g/mL single-stranded RNA equation. The A260/A280 ratio approximately 1.9 was accepted for clean RNA. First-strand cDNA was synthesized by using 2.5 μg total RNA as template, 200 pmol of each dNTP (Thermo Scientific, United States), 200 U Maxima H Minus Reverse Transcriptase (Thermo Scientific, United States) and 500 pmol random hexamer primers (Sigma-Aldrich, Hungary) in a final volume of 20 μL, and incubated for 10 min at 37 °C, followed by 45 min at 52 °C. Real-time qPCR was performed for gene expression studies, using Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Scientific, United States) in Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Hungary). The qPCR reactions were carried out with a temperature program of 10 min at 95 °C (initial denaturing), followed by 45 cycles of 15 s at 95 °C; 30 s at the annealing temperature 63 °C followed by a melting curve stage with temperature ramping from 60 to 95 °C and a final cooling for 30 s at 40 °C. The quantities of examined messenger ribonucleic acid (mRNAs) were normalized to that of 18 S ribosomal RNA, and gene expression was calculated in terms of $2^{\Delta\Delta Ct}$ method[39].

Primers

Primers were designed based on the data bank entries. For normalization of the amounts of MMP9 and TIMP1 mRNA, the 18 S RNA level was used as internal standard. MMP9 2F: 5' CTCTACACGGAGCACGGCAACG 3'; MMP9 2R: 5' CGGTGGTGGCGCACCAGCG 3'.TIMP1 2F: 5' ACAGGTTTCCGGTTCGCCTAC 3';

TIMP1 2R: 5′ CTGCAGGCAGTGATGTGCAA 3′. 18S F: 5′ GAAACGGCTACC
ACATCCAAGG 3′; 18S R: 5′ CCGCTCCCAAGATCCAACTACG 3′.

Statistical analysis
Statistical analysis was performed with Kruskal-Wallis test, and Dunn's multiple comparisons test (electron microscopic study), or one-way analysis of variance with Newman-Keuls test (Table 1 and qPCR study). Statistical analysis of RT-qPCR reactions for each animal were performed in triplicate to increase the reliability of the measurements. All analyses were carried out with GraphPad Prism (GraphPad Software, United States). A probability of $P < 0.05$ was set as the level of significance.

RESULTS

Disease characteristics of diabetic and insulin-treated diabetic rats
The general characteristics of the STZ-induced diabetic and insulin-treated diabetic, as well as the control animals are shown in Table 1. Untreated diabetic rats were characterized by a significantly reduced body weight and a markedly increased blood glucose concentration (26.23 ± 1.89 mmol/L) as compared to the sex-and age-matched controls (5.99 ± 0.19 mmol/L). In the immediate insulin replacement group, the body weight of the animals was similar to controls by the end of the experiment. The blood glucose concentration of insulin-treated rats (7.13 ± 1.37 mmol/L) remained at the control level during the 10 wk experimental period.

Morphometry of the BM surrounding myenteric ganglia
Myenteric ganglia are surrounded by a thin BM, which delimits the ganglia from the adjacent tissues. The collagen fibrils located in the extracellular space never enter into the ganglia (Figure 1A).

In controls, BM thickness was the same in the proximal and distal part of the small intestine (approximately 34-36 nm), however, a region-specific thickening was revealed in diabetic rats. The ganglionic BM was significantly thicker in the diabetic ileum relative to control values (44.04 ± 1.62 nm *vs* 34.85 ± 1.11 nm, $P < 0.0001$), whereas in the duodenum it did not exceed that of the controls (Figure 1B). Although the diabetes-related BM thickening was prevented by immediate insulin treatment in the ileum, the BM was significantly thinner (26.98 ± 0.93 nm) in this region in insulin-treated rats (Figure 1B).

Presence of MMP9 and TIMP1 immunoreactivity in the gut wall
Double-labelling fluorescent microscopy revealed MMP9 and TIMP1 immunore-activity in myenteric ganglia and their environment (Figure 2). The intensity of the fluorescent labelling varied among different structures of the gut wall: it was the lowest in the ganglia, higher in the intestinal blood vessels, and pronouncedly intense in the circular and longitudinal smooth muscle layers (Figure 2).

Quantitative changes in MMP9 expression in different cellular compartments
The expression of MMP9 was further demonstrated by gold-labelling immunoelectron microscopy in myenteric ganglia, endothelial cells of capillaries in the vicinity of these ganglia and smooth muscle cells (Figure 3). The 18 nm gold particles indicating MMP9 were often detected in cytosol, nuclei, intracellular organelles of the neuronal perikaryon (Figure 3A) and neuropil region (Figure 3B) of the ganglia, caveolar compartments, and plasma membrane of endothelial and smooth muscle cells (Figure 3C).

In control animals, the density of MMP9-labeling gold particles was significantly higher in the myenteric ganglia of the ileum than in the duodenal segment ($P < 0.001$; Figure 4A). Also, higher ileal density of MMP9 particles was observed in endothelial and smooth muscle cells (data not shown separately, but visible on Figure 5B and C).

In diabetic rats, the number of MMP9-labeling gold particles significantly decreased in the myenteric ganglia (Figure 5A), capillary endothelium (Figure 5B) and intestinal smooth muscle (Figure 5C) of the ileum. The greatest decrease has been observed in the endothelial cells of diabetics, where the number of MMP9 particles was more than half of the controls (0.75 ± 0.11 *vs* 1.66 ± 0.26). The immediate insulin treatment only partially prevented the diabetes-related alterations in MMP9 expression. However, in the diabetic duodenum, the number of MMP9 gold particles did not change in the myenteric ganglia or in the other cell types in either of the experimental groups

Table 1 Weight and glycaemic characteristics of the experimental groups
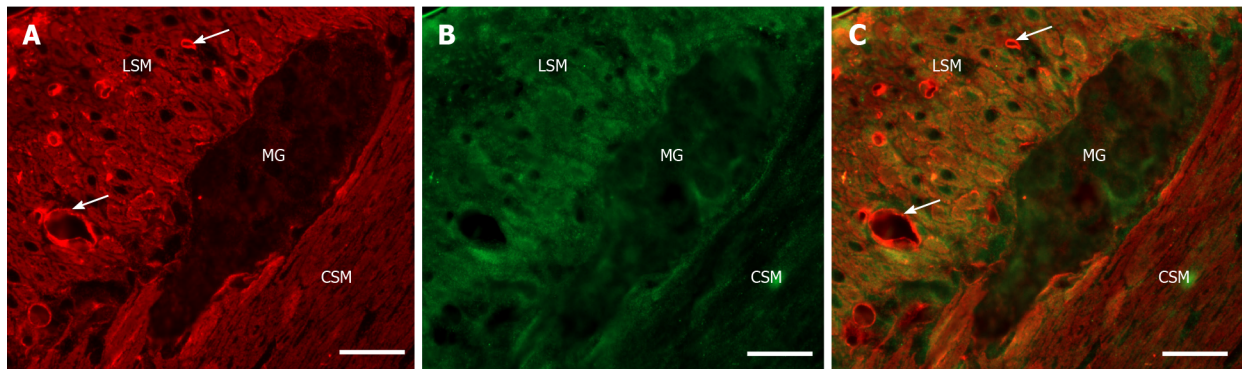
	Weight (g)		Blood glucose level (mmol/L)	
	Initial	Final	Initial	Final (average)
Controls (*n* = 4)	266 ± 18.85	460.25 ± 22.34[b]	6.48 ± 0.2	5.99 ± 0.19
Diabetics (*n* = 4)	274 ± 17.11	382.5 ± 17.69[a,c]	6.4 ± 0.24	26.23 ± 1.89[b,d]
Insulin-treated diabetics (*n* = 4)	274 ± 15.36	457.25 ± 29.33[b,e]	6.28 ± 0.09	7.13 ± 1.37[f]

[a]$P < 0.01$.

[b]$P < 0.0001$ *vs* initial.

[c]$P < 0.05$.

[d]$P < 0.0001$ *vs* final controls.

[e]$P < 0.05$.

[f]$P < 0.0001$ *vs* final diabetics. Data are expressed as mean ± SEM.

Figure 1 Representative electron micrograph of a myenteric ganglion from an insulin-treated diabetic rat. A: The myenteric ganglion is surrounded by basement membrane (BM) (arrows). Scale bar: 500 nm; B: Quantitative evaluation of BM thickness in different gut segments of control, diabetic, and insulin-treated diabetic rats. The thickening of BM surrounding myenteric ganglia remained unchanged in the duodenum, however it increased significantly in the ileum of diabetic rats. Immediate insulin treatment prevented BM thickening in the ileum. [a]$P < 0.0001$ (relative to the controls); [b]$P < 0.0001$ (between diabetics and insulin-treated diabetics).

Figure 2 Representative fluorescent micrograph of a paraffin section of myenteric ganglia from the ileum of a control rat after matrix metalloproteinase 9-tissue inhibitor of metalloproteinase 1 double-labelling immunohistochemistry. A: Matrix metalloproteinase 9 immunoreactivity is indicated in red; B: tissue inhibitor of metalloproteinase 1 immunoreactivity is shown in green; and C: The merge is depicted on. Scale bar: 20 μm. MG: Myenteric ganglia; LSM: Longitudinal smooth muscle layer; CSM: Circular smooth muscle layer; arrows-blood vessels.

(Figure 5).

Quantitative changes in TIMP1 expression and MMP9/TIMP1 ratio

TIMP1 displayed a quite low, but region-dependent expression in the myenteric

Figure 3 Representative electron micrographs subjected to matrix metalloproteinase 9 post-embedding immunohistochemistry. A: Myenteric ganglia from a control duodenum; B: A diabetic ileum; and C: Capillary endothelium and intestinal smooth muscle from a control duodenum.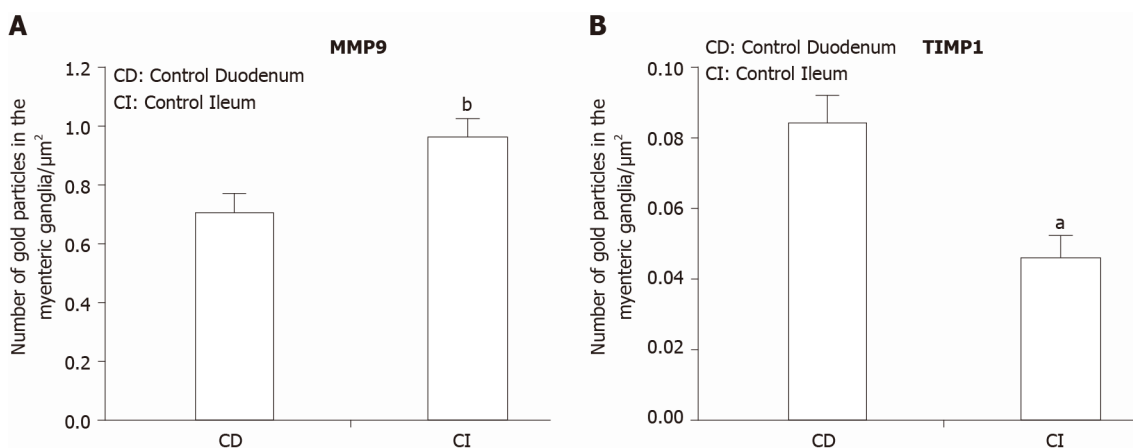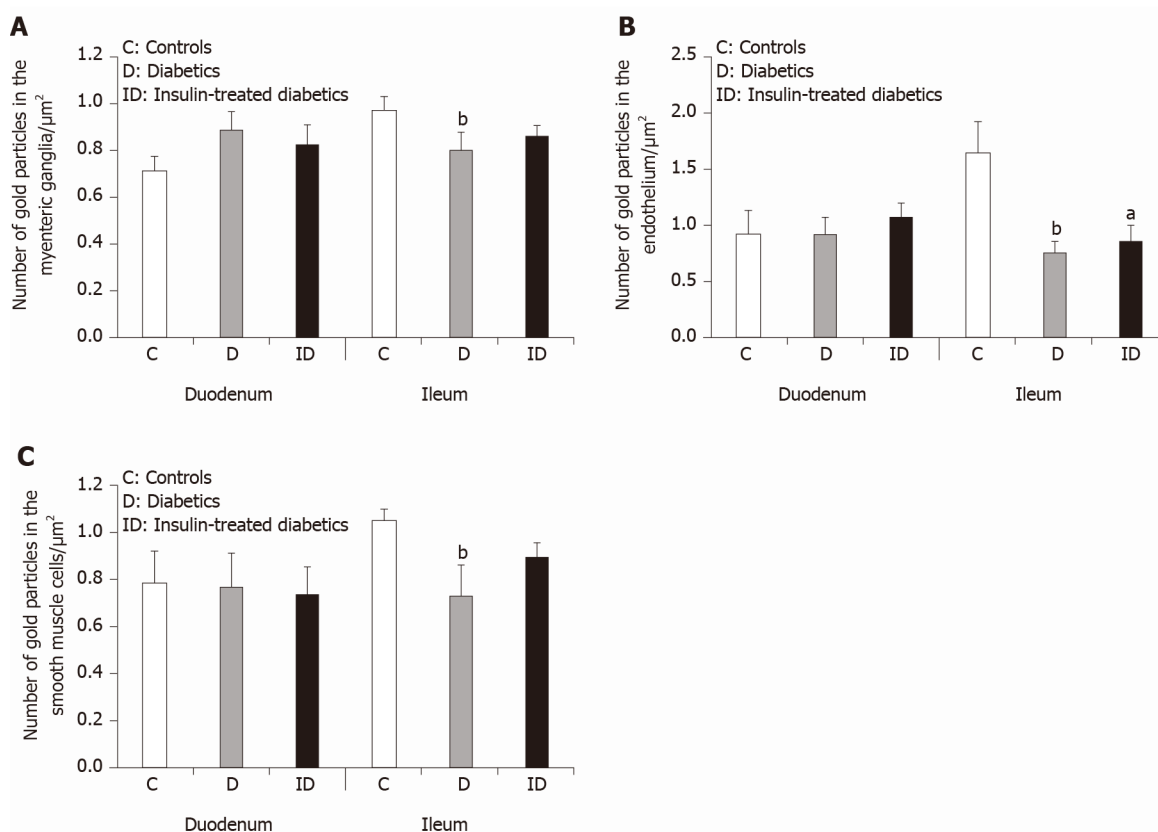 The 18 nm gold particles (arrows) indicating matrix metalloproteinase 9 were observed in cytosol, nuclei or in association with intracellular organelles and plasma membrane. Scale bars: 500 nm. P: Neuronal perikaryon; N: Nucleus; SMC: Smooth muscle cell; EC: Endothelial cell; LU: Capillary lumen.

Figure 4 Quantitative changes in MMP9 and TIMP1 expression. A: Quantitative evaluation of matrix metalloproteinase 9 (MMP9); and B: Quantitative evaluation of tissue inhibitor of metalloproteinase 1 (TIMP1) labelling gold particles in myenteric ganglia from different gut segments of control rats. In control conditions, the number of MMP9 particles was significantly higher, while TIMP1-labeling was significantly lower in the distal part of the small intestine. Data are expressed as means ± SEM. [a]$P < 0.01$ and [b]$P < 0.001$ (between control duodenum and control ileum).

ganglia of different gut segments in control conditions. The number of TIMP1-labeling gold particles was half in the ileal than in the duodenal ganglia ($P < 0.01$; Figure 4B). There were no significant differences in the distribution of TIMP1 gold particles between different intestinal regions in other control cell types (data not shown). In addition, neither the hyperglycaemia nor the immediate insulin treatment resulted in any significant alterations in the number of TIMP1-labeling gold particles in either of the cellular compartments (data not shown).

Figure 5 Quantitative changes in matrix metalloproteinase 9 expression in different cellular compartments. A: Quantitative evaluation of matrix metalloproteinase 9 (MMP9)-labeling gold particles in myenteric ganglia; B: Capillary endothelium; and C: Intestinal smooth muscle cells from different gut segments of control, diabetic, and insulin-treated diabetic rats. In diabetics, the number of MMP9-labeling gold particles was significantly decreased in all cellular compartments of the ileum, while it was unchanged in the duodenum relative to controls. The number of gold particles was closer to the control values after immediate insulin treatment. Data are expressed as means ± SEM. [a]$P < 0.05$ and [b]$P < 0.01$ (relative to controls).

However, a region-specific MMP9/TIMP1 ratio was observed in the myenteric ganglia. While the MMP9/TIMP1 ratio remained unchanged in the duodenum, the decrease in the ratio was nearly 50% in the ileal ganglia of diabetic rats (Figure 6). The MMP9/TIMP1 ratio was more than double in the duodenum, while it was close to the control value in the ileum in the insulin-treated group (Figure 6).

Quantitative alterations in mmp9 and timp1 mRNA expression

The expression level of MMP9 mRNA markedly increased in both gut segments under chronic hyperglycaemic conditions. The rate of induction was approximately 5.5-fold in tissue homogenates prepared from the diabetic duodenum, and approximately 7.0-fold in the diabetic ileum relative to controls and normalized to the endogenous 18S RNA as reference (Figure 7).

In parallel, TIMP1 mRNA expression was highly upregulated (approximately 5-fold) in tissue homogenates originating from the ileum, while it remained unchanged in duodenal homogenates of diabetic rats (Figure 7).

DISCUSSION

In accordance with our principle findings that diabetic myenteric neuropathy is intestinal region-dependent[4], and that the neuronal microenvironment is also suffering from a series of strictly region-specific diabetic damages[12,40], the present study provides more evidence of gut segment-specific, diabetes-related alterations of the ECM biology in myenteric ganglia, intestinal capillaries and smooth muscle of the gut wall.

We have shown for the first time that the BM surrounding myenteric ganglia is regionally thickened along the small intestine. The thickness was increased by more than 25% in the ileum, while it was unchanged in the duodenum of diabetic rats.

Figure 6 The ratio of matrix metalloproteinase 9 and tissue inhibitor-labeling gold particles in myenteric ganglia of different gut segments of control, diabetic, and insulin-treated diabetic rats. In diabetic rats, the matrix metalloproteinase 9/tissue inhibitor of metalloproteinase 1 ratio was not altered in duodenal, but was markedly decreased in ileal ganglia. The immediate insulin replacement did not restore the equilibrium ratio observed in controls.

Figure 7 Fold change in the messenger ribonucleic acid levels of matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 genes, measured by real-time fluorescence-based quantitative polymerase chain reaction using the $2^{\Delta\Delta Ct}$ method. Data sets are presented as the fold change in gene expression normalized to the endogenous reference (RNA18S) and relative to the untreated controls. Data are expressed as means ± standard deviation. [a]$P < 0.001$ and [b]$P < 0.0001$.

Using the same type 1 diabetic rat model, we formerly demonstrated that the endothelial BM of intestinal capillaries supplying the enteric ganglia displayed similar structural alterations[12]. The thickening and separation of capillary BM was pronounced in the distal part of the gastrointestinal tract, but not in the duodenum of diabetics[12], which suggests optimal and very stable conditions in the duodenum while a more susceptible microenvironment is plausible in the ileum along the proximal-distal axis of the gut. Other findings include increased amount of ECM proteins (laminin-1 and fibronectin), as well as BM thickening of the small intestinal smooth muscle cells in diabetic rats[41], and also significant thickening of perineurial cell BM and loss of myelinated nerve fibres in diabetic peripheral neuropathies[42-44]. The immediate insulin replacement prevented diabetes-related BM thickening surrounding the ileal myenteric ganglia, as it inhibited the BM thickening of capillary endothelium[12] or reversed the hyperglycaemia-induced ECM accumulation in the intestinal smooth muscle layers[41]. Among the underlying mechanisms of ECM accumulation resulting in both ganglionic and endothelial BM thickening, the matrix degrading metalloproteinases have become the focus of the present study. We have shown that MMP9 and TIMP1 is present in myenteric ganglia and their environment using fluorescent and electron microscopy. The ultrastructural localization of these markers within the cytoplasm and nuclei of the enteric neurons, endothelial and smooth muscle cells was in agreement with other findings[45,46]. The nuclear localization of MMPs may have a role in regulation the activity of DNA-repairing and apoptotic proteins[47,48].

Quantitative immunogold labelling showed that the MMP9 particle density increased, while the TIMP1 particle density decreased in myenteric ganglia along the proximal-distal axis of the small intestine under control conditions. In addition to this opposite tendency, we observed that the particle density of TIMP1 was an order of magnitude lower than that of MMP9. In diabetic rats, the MMP9-indicating gold particles decreased in all cell types of the ileum, however, they remained unchanged in all duodenal compartments. Moreover, the MMP9/TIMP1 ratio was also decreased only in the ileal ganglia, but not in the duodenum. Immediate insulin replacement prevented diabetic alterations only in part as it was described in other studies[4,5,12].

The region-dependent BM thickening and changes in MMP production in different cellular compartments correlate well with each other. In the diabetic ileum, the decrease in MMP9 expression both in myenteric ganglia, and endothelial and smooth muscle cells suggests a decreased breakdown of ECM components resulting in ECM accumulation and thickening of BM around the myenteric ganglia and also the intestinal capillaries[12]. However, in the diabetic duodenum, where MMP9 production remained optimal in all cell types, the thickness of ganglionic and endothelial BM also remained unchanged[12].

Although we did not observe significant changes in the distribution of TIMP1 protein-detecting gold particles in different cell types under diabetic conditions and after insulin treatment, the segment-specific induction of both MMP9 and TIMP1 mRNA expression was demonstrated. Meanwhile, the MMP9 induction was not accompanied by altered TIMP1 expression in the duodenal tissue homogenates of diabetics, however, in the ileal samples, a 7-fold increase in MMP9 mRNA level was detected, along with a great upregulation in TIMP1 mRNA expression. The regulation of MMPs/TIMPs expression and activity is complex, involving both transcriptional and post-transcriptional mechanisms using multiple signal pathways, microRNA modulation, post-translational modifications and extracellular inhibition[21,49-51]. Based on these findings, we presume that despite of the MMP9 transcriptional alterations, due to post-transcriptional modifications[49], the balance in the production of MMP9 and TIMP1 proteins was retained in the duodenum, which contributes to the maintenance of optimal ECM dynamics in this segment. In contrast, in the ileum, not only MMP9 but also TIMP1 transcription was highly upregulated, resulting in MMP9 protein underproduction and ECM accumulation. Inconsistent alterations of MMP9 mRNA and protein expression presuming post-transcriptional regulation were also observed in diabetic nephropathy[52]. Moreover, upregulated microRNA production resulted in decreased MMP9 expression, which in turn contributed to renal fibrosis in diabetic kidney[52,53]. Decreased matrix degradation due to increased MMP mRNA level, but decreased activity was also documented in diabetic nephropathy[17,54], modulated by advanced glycation end products[55]. Increased fibrosis was also demonstrated in MMP knockout mice[18]. In contrast, some studies report that MMP overexpression promotes renal fibrosis due to a possible interplay with TIMPs[51,56], while others revealed that MMP2 and MMP9 triggered mitochondrial damage and apoptotic processes in retinal capillaries[57-59].

However, it certainly seems that the imbalance in MMP/TIMP ratio[60,61] caused by various molecular mechanisms disturbs the equilibrium of ECM degradation and turnover and contributes to several inflammatory processes, as well as vascular or neuronal damage, which requires further investigations.

CONCLUSION

Overall, in the present study, we provided evidence that the region-dependent thickening of ganglionic basement membrane is closely related to the regionally decreased MMP9 expression in myenteric ganglia and its environment, coinciding with the intestinal region-specific enteric neuropathy in type 1 diabetes.

ARTICLE HIGHLIGHTS

Research background

The diabetic damage of enteric neurons and intestinal capillaries supplying the enteric ganglia are strictly intestinal region dependent. Therefore, the underlying molecular differences in the neuronal environment should be more emphasized.

Research motivation

To prove the presence of essential regional differences in the neuronal milieu which may explain the gut segment-specific enteric neuropathy and vascular dysfunction.

Research objectives

To reveal the impact of diabetes and immediate insulin treatment on the thickness of basement membrane (BM) surrounding myenteric ganglia, as well as the expression of matrix metalloproteinase 9 (MMP9) and its tissue inhibitor of metalloproteinase 1 (TIMP1) which are key players in regulating extracellular matrix dynamics.

Research methods

Electron microscopic morphometry, fluorescent and gold-labelling immunohisto-chemistry and quantitative polymerase chain reaction were applied to study the myenteric ganglia and their environment in the different gut segments of diabetic, insulin-treated diabetic and control rats.

Research results

In the diabetic ileum, the ganglionic BM was significantly thickened which was prevented by insulin treatment. These changes were also reflected in a decrease in MMP9/TIMP1 ratio in ileal myenteric ganglia. However, in the duodenum of diabetics neither the ganglionic BM thickness nor the MMP9/TIMP1 ratio were changed.

Research conclusions

Regionally decreased MMP9 expression in ganglia and region-dependent ganglionic BM thickening correlate well with intestinal segment-specific enteric neuropathy.

Research perspectives

Based on these findings in type 1 diabetic rat model, we are planning to expand our investigations to type 2 diabetes in the future.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Observational Study

Relationships between emissions of toxic airborne molecules and type 1 diabetes incidence in children: An ecologic study

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Abstract

BACKGROUND

Type 1 diabetes originates from gene-environment interactions, with increasing incidence over time.

AIM

To identify correlates of childhood type 1 diabetes in European countries using an ecological approach. Several environmental variables potentially influencing the onset of type 1 diabetes have been previously evaluated. However, the relationships between epidemiologic data and exposure to toxic airborne molecules are scarcely studied.

METHODS

We employed an ecological model to explore, in a wide time period (1990-2018), associations between type 1 diabetes incidence in 19 European countries (systematic literature review) and the nationwide production of five widely diffused air pollutants: particulate matter < 10 μm (PM10), nitrogen oxides (NO), non-methane volatile organic compounds (VOCs), sulphur oxide (SO_2), and ammonia.

RESULTS

Data confirm a raising incidence of type 1 diabetes in 18 out of 19 explored countries. The average difference (last *vs* first report, all countries) was +6.9 × 100000/year, with values ranging from -1.4 (Germany) to +16.6 (Sweden) per 100000/year. Although the overall production of pollutants decreased progressively from 1990 to 2018, type 1 diabetes incidence was positively associated with the nationwide emissions of PM10, VOCs, and NO but not with those of

SO_2 and ammonia. Type 1 diabetes incidence was significantly higher in countries with high emissions than in those with low emissions of PM10 (27.5 ± 2.4 *vs* 14.6 ± 2.4 × 100000 residents, respectively), VOCs (24.5 ± 4.4 *vs* 13.2 ± 1.7 × 100000 residents, respectively), and NO (26.6 ± 3 *vs* 13.4 ± 2.4 × 100000 residents, respectively), but not of SO_2 or ammonia.

CONCLUSION

Evidence justify further studies to explore better links between long-term air quality and type 1 diabetes onset at the individual level, which should include exposures during pregnancy. In this respect, type 1 diabetes could be, at least in part, a preventable condition. Thus, primary prevention policies acting through a marked abatement of pollutant emissions might attenuate future type 1 diabetes incidence throughout Europe.

Key Words: Type 1 diabetes; Epidemiology; Air pollution; Particulate matter; Nitrogen oxide; Non-methane volatile organic compounds

Core Tip: The environment has a role in the onset of type 1 diabetes. Possible associations include pollutants that are, however, scarcely explored. We evaluated with an ecologic approach associations between the incidence of type 1 diabetes in children and the global emissions of specific air pollutants in 19 European countries, during three decades. We showed that the incidence of type 1 diabetes is associated with the emissions of particulate matter < 10 μm, non-methane volatile organic compounds, and nitrogen oxides. Results allow us to speculate that type 1 diabetes is, at least in part, a preventable condition, with implications in terms of primary prevention.

INTRODUCTION

The onset of type 1 diabetes is linked with pathogenic processes involving formation of autoantibodies, islet specific T-cells, and progressive inflammatory destruction of the beta-cells[1]. These pathways result from complex interactions between genetic[2,3] and environmental factors[4], which also involve epigenetic mechanisms [5].

Epidemiologic studies reveal a progressive increase in the incidence of type 1 diabetes in children, which doubled over the last 20 years, with a 3.4% increase per annum[6]. In European countries, the incidence of type 1 diabetes in pediatric age shows an increment rate of about 3% per year[7], but different trends occur in different geographical areas[7].

According to preliminary observations, the epidemiologic increment of type 1 diabetes could be due, at least in part, to an unhealthy environment. In particular, type 1 diabetes onset has been linked with the concentration of airborne molecules (introduced with breath), and with chemicals introduced by oral ingestion or direct cutaneous contact (endocrine disrupting chemicals)[8].

These molecules can also operate during the *in utero* life[9,10], increasing the risk of developing type 1 diabetes mainly through immune alterations[11-13] and damage to pancreatic beta-cells[14].

Preliminary evidence, in particular, suggest a role for airborne pollutants as ozone[10], nitrogen oxides (NO)[9], particulate matter[8,15], sulphate, nitrates, nitrites, N-nitroso compounds, persistent organic pollutants, heavy metals, and volatile organic compounds (VOCs)[8].

However, so far, the possibility that long-term exposure to a large number of diffused air pollutants may affect the incidence of type 1 diabetes in children has not been fully confirmed.

We aimed to identify correlates of childhood type 1 diabetes in European countries using an ecological approach. The methodology links temporal trends of the global nationwide production of toxic airborne molecules with type 1 diabetes incidence in children living in the same European areas.

MATERIALS AND METHODS

We performed a systematic review of literature (PubMed) to collect and examine available data about the incidence of type 1 diabetes in pediatric age (0-15 years) in 19 European countries (Supplementary Tables 1 and 2). All publications until December 4, 2020 were considered. Papers were selected by the following criteria: (1) the study period was ≥ 2 years; (2) the study considered the overall age-standardized incidence of type 1 diabetes (*per* 100000 per year) in at least one European country and in the age range 0-15 years; (3) the study was based on a nationwide dataset; (4) study periods starting from the year 1990. This time limit depended on the lack of information about countrywide pollutant production before 1990 (see below); and (5) studies published in English language.

In the selected studies, the incidence of type 1 diabetes was assessed, on average, on a time period of 17.8 years (range 4-28 years), from 1990 to 2018 (Supplementary Table 2). In the case of different studies examining similar time periods in the same country, data from the study with the shortest period were excluded from the analysis, in order to avoid time overlapping and correlation bias.

Data about pollutant emissions were derived from the European Environmental Agency and Eurostat database (https://ec.europa.eu/eurostat/databrowser/view/ENV_AIR_EMIS__custom_210633/default/table?lang=en, last update 25 November 2020), and measured as tonnes of emissions per year, considering total sectors of emissions for each pollutant and for the national territory, in the period 1990-2018. Average emissions in selected periods (corresponding to type 1 diabetes incidence time periods in each country) were thereafter calculated for the following pollutants: Particulate matter < 10 μm (PM10), NO, non-methane organic VOCs, sulphur oxide (SO$_2$), and ammonia. Correlations between the global amount of pollutant emissions and type 1 diabetes incidence were checked for each country/time period by Spearman's rank correlation coefficient.

We categorized each pollutant according to tertiles of emissions in the entire study period (low, medium, and high pollutant emissions). To calculate odds ratios and confidence intervals for type 1 diabetes incidence associated with emissions of specific pollutants, we fit separate logistic regression models with type 1 diabetes incidence as the dependent variable, and tertiles of each pollutant as the independent variable. Kruskal-Wallis analysis of variance by ranks followed by multiple-comparison *Z*-value test or Mann-Whitney *U*-test were employed to compare differences among groups, as appropriate. $P < 0.05$ were considered statistically significant for all analysis. Graphic representation of data is provided by SigmaPlot software (https://systatsoftware.com/products/sigmaplot/). Statistical analyses were performed with NCSS10 Statistical Software (NCSS, LLC, Kaysville, UT, United States).

RESULTS

Altogether, in 19 European countries, 18 studies considering the incidence of type 1 diabetes met the inclusion criteria and were considered in the analysis (Supplementary Table 1).

Figure 1 shows time variations in type 1 diabetes incidence across Europe in each of the explored countries, in the whole observation period. During the explored time interval, type 1 diabetes incidence increased in all explored countries, with the exception of Germany.

The average difference (last *vs* first report, all countries) was +6.9 × 100000/year, with values ranging from -1.4 (Germany) to +16.6 (Sweden) per 100000/year (Figure 2).

Considering the whole group of explored countries, the global, nationwide production of pollutants decreased progressively from 1990 to 2018 (Figure 3A), with significant reductions recorded in the case of VOCs, PM10, SO$_2$, and NO but not for

Figure 1 Age-standardized incidence of type 1 diabetes (× 100000) in children from 19 European countries, according to time. Incidence data were recorded at various time intervals in the period 1990-2018 and were combined. Data for boys and girls have been pooled.

Figure 2 Type 1 diabetes incidence variation in the period 1990-2018. Data are presented as absolute difference between the recorded incidence (× 100000) in the last *vs* first report, in each country. Malta was excluded from the analysis, since type 1 diabetes incidence was assessed in a single report exploring a single time period. T1D: Type 1 diabetes.

ammonia (Figure 3B).

The incidence of type 1 diabetes in European countries was significantly positively correlated with national PM10 emissions (ρ = 0.32, *P* = 0.004), VOCs (ρ = 0.35, *P* = 0.001), and NO (ρ = 0.44, *P* = 0.0001) but not with those of SO_2 and ammonia (*P* = NS, data not shown).

When countries were stratified according to tertiles of pollutants emitted in the entire period (Supplementary Table 3), the odds of elevated type 1 diabetes incidence were significantly higher in countries in the medium and high tertiles of PM10, VOCs,

Figure 3 Pollutant emissions. A: Time variations in the nationwide production of pollutants (tonnes per year, total sector of emissions for each pollutant) recorded in 19 European countries from 1990 to 2018. Symbols indicate average values; B: Percent decrement (2018 *vs* 1990) in the nationwide production of pollutants ([a]*P* < 0.05, last *vs* first year, Mann-Whitney). NO: Nitrogen oxides; PM10: Particulate matter < 10 μm; SO$_2$: Sulphur oxide; VOCs: Volatile organic compounds.

and NO production (but not SO$_2$ or ammonia, data not shown) than in those in the low production group (Table 1 and Figure 4A). Mean incidence of type 1 diabetes was significantly higher in countries with high production than in countries with low production of PM10, VOCs, and NO (Supplementary Table 3, Table 2 and Figure 4B). The incidence of type 1 diabetes was similar when countries were compared in terms of SO$_2$ and ammonia production (data not shown).

DISCUSSION

The present study examined type 1 diabetes incidence during about three decades (1990-2018) and reports a progressive increase in the majority of the 19 explored European countries. We employed an ecologic approach and found an association between type 1 diabetes incidence and the global burden of anthropogenic emissions of three widely diffused air pollutants (namely PM10, non-methane VOCs, and NO), with increased odds of high incidence in countries with the highest pollutant emissions.

We confirm previous observations about the worldwide rise in type 1 diabetes incidence with time in pediatric age[7,16-19].

The selected papers cover a time window of about 18 years. Although genetic susceptibility is a well-known risk factor for the onset of type 1 diabetes[2,3], the relatively short time interval does not explain the increase in pediatric type 1 diabetes incidence simply by shifts in individual genetic susceptibility. A larger time window is usually needed to establish genetic changes at a population level. Indeed, prior studies found a rise in type 1 diabetes incidence associated with unchanged[3] or even decreased[20] frequency of major genetic risk factors for type 1 diabetes. Furthermore, only a minority of genetically susceptible children progress to clinical disease[21-23], and the concordance rate among monozygotic twins ranges from 13% to 68%, and is approximately 6% in siblings[21-23].

These elements therefore point to a critical role for environmental factors that also operate during pregnancy[4,23,24], as well as for mechanisms acting through the interplay gene-environment[25].

The relationships between the onset of type 1 diabetes and the environment have investigated a number of factors, including deprivation[26]. None among these factors, however, is convincingly implicated in the etiology of type 1 diabetes as a chronic autoimmune disease[23,24,26,27]. Environmental pollution is still scarcely explored, despite the link with several autoimmune diseases[28,29], namely rheumatic diseases[30], thyroid diseases[31], and systemic lupus erythematosus[32].

Table 1 Odds ratios and 95% confidence intervals of type 1 diabetes incidence in 19 European countries stratified according to tertiles of particulate matter < 10 μm, volatile organic compounds, and nitrogen oxides emissions

Tertiles of emission	PM10	VOCs	NO
Reference, low	1	1	1
II tertile, medium	1.08 (1.01-1.15)	1.19 (1.1-1.29)	1.12 (1.04-1.21)
III tertile, high	1.10 (1.04-1.18)	1.16 (1.05-1.28)	1.15 (1.05-1.24)

Observation period: Years 1990-2018. NO: Nitrogen oxides; PM10: Particulate matter < 10 μm; VOCs: Volatile organic compounds.

Table 2 Average incidence of type 1 diabetes (× 100000) in 19 European countries stratified according to tertiles of particulate < 10 μm, volatile organic compounds, and nitrogen oxides emissions

	PM10	VOCs	NO
Reference, low	14.6 ± 2.4	13.2 ± 1.7	13.4 ± 2.4
II tertile, medium	21.3 ± 2.2	28.4 ± 1.8[a]	23.6 ± 1.9[a]
III tertile, high	27.5 ± 2.4[b]	24.5 ± 4.4[a]	26.6 ± 3[a]

Observation period: Years 1990-2018. Data are means ± SE.

[a]$P < 0.05$ *vs* countries in the low tertile of emissions.

[b]$P < 0.05$ *vs* countries in the low and medium tertile of emissions (Kruskal-Wallis analysis of variance by ranks followed by multiple-comparison Z-value test). NO: Nitrogen oxides; PM10: Particulate matter < 10 μm; VOCs: Volatile organic compounds.

The pathogenic relationship environment-autoimmune diseases might also exist in pediatric age[33-36]. In particular, toxicants strongly affect the development of the immune system also during *in utero* life. In newborns, living in a highly polluted urban area is associated with lower percentage of CD4+ T-lymphocytes and a lower CD4+ / CD8+ ratio, but higher percentage of natural killer cells[37]. In the same population, prenatal maternal exposure to polycyclic aromatic hydrocarbons and fine particulate matter was associated with a lower percentage of CD3+, CD4+, and CD8+ T-lymphocytes and with a higher percentage of CD19+ B-lymphocytes[38].

Among environmental toxics, bisphenol A[39-42], higher intake of nitrates, nitrites, N-nitroso compounds, and persistent organochlorine pollutants[43-45] are potentially associated with the onset of type 1 diabetes. Limited evidence[43,44] exist for heavy metals like chromium[46], cadmium[47], and lead[48].
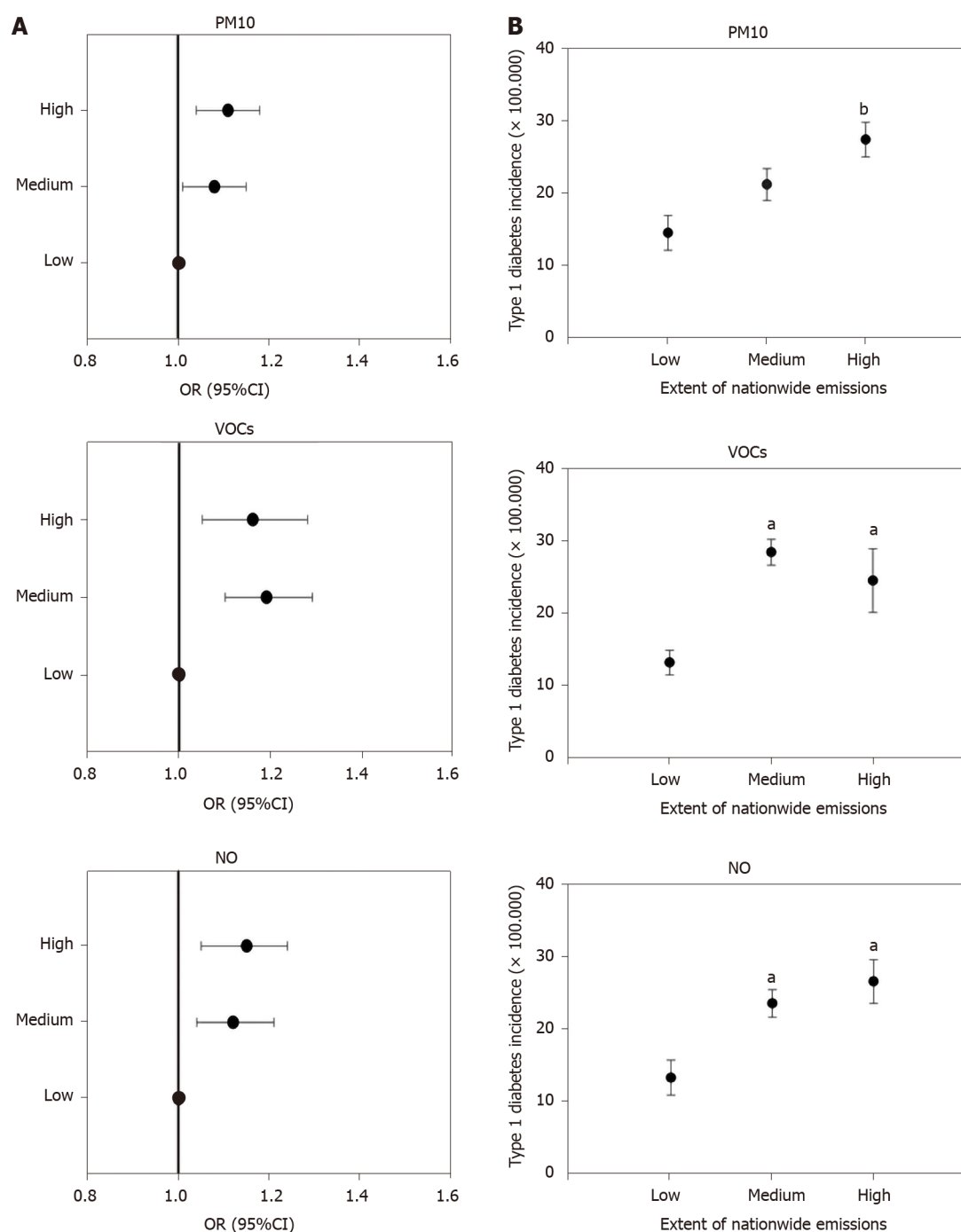
Air pollution is a heterogeneous mixture of molecules present in gases and solid particles, each having its own potential pathogenic effect. This study found a possible association between country emissions of PM10, NO, VOCs, and type 1 diabetes incidence. Of note, maternal exposure before and during pregnancy to PM10, NO[49], and VOCs[49,50] affects blood lymphocyte immunophenotype distribution in newborns, even at low air concentrations.

Our results about PM10 and type 1 diabetes incidence are in line with previous epidemiological observations. A large cohort study in Bavarian children found that exposure to high levels of PM10 and nitrogen dioxide can accelerate the onset of type 1 diabetes and the risk of developing type 1 diabetes in children with less than 5 years of age. Mechanisms involve a more severe inflammatory state[51]. Overall, results indicate that the exposure to PM10 is contributing to the increased incidence of type 1 diabetes in pediatric age[8], in particular in children with less than 5 years of age[52].

Non-methane VOCs are widely diffused indoor and outdoor pollutants. Although systematic studies are lacking, there are evidence showing that *in utero* exposure to VOCs affects the immune system in animal models[53] and in newborns[49,50]. More studies should explore the role of VOCs on the pathogenesis and on the epidemiological risk of developing type 1 diabetes.

The relationships between chronic exposure to NO and the risk of type 1 diabetes is also scarcely explored. A previous report from a large cohort study in Swedish children found a significant association between NO exposure during the third trimester of pregnancy and offspring type 1 diabetes[9]. Of note, the amount of NO emission is among the main factors causing ozone pollution[54]. Studies found that cumulative exposure to ozone and sulfate in ambient air increased the risk of

Figure 4 Odds ratios, 95% confidence interval, and incidence of type 1 diabetes in 19 European countries grouped according to tertiles of emissions of particulate matter < 10 μm, volatile organic compounds, and nitrogen oxides. A: Odds ratios and 95% confidence intervals of type 1 diabetes incidence in 19 European countries grouped according to tertiles of particulate matter < 10 μm, volatile organic compounds, and nitrogen oxides (NO) emissions (total sector of emissions for each pollutant in each country). Values were calculated by logistic regression models, with type 1 diabetes incidence as the dependent variable, and tertiles of each pollutant (low, medium and high pollutant emissions) as the independent variable; B: Type 1 diabetes incidence in 19 European countries grouped according to tertiles (low, medium, high) of emissions of particulate matter < 10 μm, volatile organic compounds, and nitrogen oxides. The amount of pollutant emissions and the average type 1 diabetes incidence were calculated for each country in the same time period. Data are means ± standard error. [a]$P < 0.05$ *vs* countries in the low tertile of emissions (Kruskal-Wallis analysis of variance by ranks followed by multiple-comparison Z-value test); [b]$P < 0.05$ *vs* countries in the low and medium tertile of emissions. CI: Confidence interval; NO: Nitrogen oxides; OR: Odds ratios; PM10: Particulate matter < 10 μm; VOCs: Volatile organic compounds.

developing type 1 diabetes in pediatric age[52,55]. The role of ozone exposure as a risk factor for type 1 diabetes has been also confirmed by a recent retrospective-population based cohort study, suggesting an increased incidence of type 1 diabetes in children aged 0-5 years exposed to high ozone levels during the prenatal period (first trimester of pregnancy)[10].

An additional aspect emerging from this study was the apparent contradiction between the increased incidence of type 1 diabetes over time and the progressive nationwide decrement of pollutants. It should be underlined that, in the whole group of explored countries, the statistical association between the type 1 diabetes incidence and the global burden of pollutants (*i.e.* tonnes of emissions per year) does not express a temporal trend. The analysis correlates the type 1 incidence recorded in a specific period, with the burden of pollutants emitted in the same explored period. From this point of view, the countries with the highest emissions of PM10, VOCs, and NO (*i.e.* nationwide emissions in the II and III tertiles) have the highest incidence of type 1 diabetes in corresponding time periods. This finding is confirmed by logistic regression models exploring the odds of elevated type 1 diabetes incidence in countries divided according to tertiles of emissions and by an analysis of variance exploring differences between the average incidence of type 1 diabetes in countries with low, medium, and high production of pollutants.

The decreasing trend in the emission of pollutants observed over time was significant for all explored pollutants, with the exclusion of ammonia. Of note, we found no significant association between type 1 diabetes incidence and SO_2 (the pollutant with the highest percent decrement in the examined period, *i.e.* -93%). The possibility exists that the decreased production of VOCs, PM10, and NO (about -60% in 2018 *vs* 1990) might be still insufficient to generate beneficial effects in terms of global type 1 diabetes incidence. In addition, previous epidemiologic findings based on other diseases could not identify a definitive threshold linking air concentration of particulate matter[56] and NO[57] with health effects. Biological effects might also occur during moderate exposure to multiple pollutants (cumulative effect).

This study has some limitations. Firstly, the analysis included children with age comprised between 0 and 15 years, as a whole group. Type 1 diabetes is a heterogeneous disease in terms of epidemiologic findings, and different trends exist in children with early onset of disease, as compared to children diagnosed type 1 diabetes in older ages[8]. However, the wide time period in the present study (about 18 years) might partly limit the bias from different epidemiologic trends in different pediatric age groups. Additional surveys should better explore the link between air pollution and type 1 diabetes incidence in different age classes, employing a specific study design (case-control or cohort studies). Secondly, the present study used an ecological approach. This methodology can only indicate the existence of ecological associations, which not necessarily point to pathogenic associations between explored pollutants and type 1 diabetes onset at an individual level. Further studies are needed to examine in details pathophysiological and epidemiological links between individual exposure to PM10, NO, and VOCs and the onset of type 1 diabetes. Future analyses, in particular, should comprehensively consider *in utero* exposures, epigenetic mechanisms, and individual variables exploring other known risk factors of type 1 diabetes as genetic factors, viral infection history, family history, individual diet, and lifestyle. Finally, the present study was not designed to explore time variations in type 1 diabetes incidence according to temporal trends of emission in each country. Studies conducted at a national level could correlate local epidemiologic and environmental data on a wide time window, possibly in different age classes[8]. Although genetic factors seem to play a limited role in the epidemiological variations of type 1 diabetes incidence[21-23], nation-based cohort or case-control studies should also allow comparisons between subgroups with similar genetic background but living in different countries.

CONCLUSION

In conclusion, the present study confirms the increasing epidemiologic trend of type 1 diabetes in pediatric age in European countries. Results point, in particular, to the association with the global burden of emissions of specific environmental pollutants (PM10, NO, VOCs). We advocate further studies exploring additional links between long-term air quality and type 1 diabetes onset. Such surveys should employ specific models and should consider exposures during pregnancy. We speculate that type 1 diabetes is, at least in part, a preventable condition and that primary prevention policies might attenuate future type 1 diabetes incidence throughout Europe, by marked abatement of pollutant emissions.

ARTICLE HIGHLIGHTS

Research background

Type 1 diabetes onset depends on gene-environment interactions, and several reports show, worldwide, an increased incidence of type 1 diabetes over time.

Research motivation

The effect of environmental factors on type 1 diabetes incidence in pediatric age is still incompletely unexplored.

Research objectives

To correlate the incidence of childhood type 1 diabetes in European countries with the global, nationwide production of toxic airborne molecules.

Research methods

We employed a systematic literature review to explore type 1 diabetes incidence in pediatric age in 19 European countries (time period: 1990-2018). We therefore applied an ecological study design to explore possible associations with the nationwide production of five widely diffused air pollutants: Particulate matter < 10 μm (PM10), nitrogen oxides (NO), non-methane volatile organic compounds (VOCs), sulphur oxide (SO_2), and ammonia.

Research results

A raising incidence of type 1 diabetes was evident in 18 out of 19 countries. Considering the whole group of countries, type 1 diabetes incidence was associated with the nationwide emissions of PM10, NO, non-methane VOCs, but not with those of SO_2 and ammonia.

Research conclusions

The global burden of emission of specific air pollutants is associated with type 1 diabetes incidence. The study design employed in the present study can only indicate the existence of ecological associations and does not necessarily point to specific pathogenic links. However, results suggest the possibility that type 1 diabetes could be, at least in part, a preventable condition.

Research perspectives

Further studies conducted with specific models are needed to explore better the pathogenic links between type 1 diabetes, air pollutants and other known risk factors of disease as genetic factors, viral infection history, individual diet, and lifestyle.

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