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AIM AND SCOPE

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INDEXING/ABSTRACTING

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REVIEW

Effects of antidiabetic drugs on epicardial fat

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Abstract

Epicardial adipose tissue is defined as a deposit of adipocytes with pathophysiological properties similar to those of visceral fat, located in the space between the myocardial muscle and the pericardial sac. When compared with subcutaneous adipose tissue, visceral adipocytes show higher metabolic activity, lipolysis rates, increased insulin resistance along with more steroid hormone receptors. The epicardial adipose tissue interacts with numerous cardiovascular pathways *via* vasocrine and paracrine signalling comprised of pro- and anti-inflammatory cytokines excretion. Both the physiological differences between the two tissue types, as well as the fact that fat distribution and phenotype, rather than quantity, affect cardiovascular function and metabolic processes, establish epicardial fat as a biomarker for cardiovascular and metabolic syndrome. Numerous studies have underlined an association of altered epicardial fat morphology, type 2 diabetes mellitus (T2DM) and adverse cardiovascular events. In this review, we explore the prospect of using the epicardial adipose tissue as a therapeutic target in T2DM and describe the underlying mechanisms by which the antidiabetic drugs affect the pathophysiological processes induced from adipose tissue accumulation and possibly allow for more favourable cardiovascular outcomes though epicardial fat manipulation.

Key words: Epicardial fat; Adipose tissue; Type 2 diabetes mellitus; Antidiabetic drugs

Core tip: In this review, we aim to create a concise overview of the pathophysiology concerning the epicardial fat deposits on a type 2 diabetic individual, while, delving into the intricacies of each antidiabetic drug and exploring the manner by which it interacts with visceral fat accumulation in the sub-pericardial space.

INTRODUCTION

Subcutaneous (SCAT) and visceral adipose tissue (VAT) are two extremely heterogenous tissue types, differentiated by anatomical, molecular, cellular, physiological and clinical characteristics[1]. Researchers have suggested that the variation of composition and function of the two tissue types is induced very early in the tissue developmental pathway, as a result of adipose stem cell distinction[2]. VAT has an anatomically distinct distribution in the mesentery and omentum, when compared to SCAT that is mainly located in the femerogluteal area, back and abdominal wall[1]. As a result of the anatomical differences, vascularization and innervation vary between the tissues, with VAT having superior nerve and vascular networks, as well as draining into the portal system of veins. Based on the aforementioned anatomical link, the "portal theory" of metabolic inflammation states that free fatty acids and pro-inflammatory molecules from VAT, interact with the liver, promoting hepatocellular dysfunction in the form of insulin resistance and steatosis[3]. The dissimilarity in cellular composition between SCAT and VAT is a result of divergent ratio of large to small adipocytes between the two tissues. Large, metabolically dysfunctional, adipocytes, predominate in VAT, while SCAT is mainly composed by small adipocytes with higher free fatty acids and triglycerides capacity and increased insulin sensitivity[4,5]. The signaling pathways activated in the two tissue types vary due to a shift in receptor distribution and adipokine synthesis[1]. Glucocorticoid and androgen receptors present with a higher density in VAT while oestrogen receptors are more active in SCAT. Adrenergic signaling patterns are distinct for the two cell populations, with VAT being more β_3- and α_2- adrenoreceptor sensitive[6]. The biologically active molecules produced by the adipose tissue, referred to as adipokines, are formed and released at different rates between VAT and SCAT. Adipokines are the basis of adipose tissue participating in and regulating endocrine and paracrine funtions[7]. The diversity of adipokines is directly linked to sympathetic excitation, metabolic regulation, including insulin sensitivity and appetite, inflammatory response and other homeostatic mechanisms. Some of the most prominent members of this family, as far as metabolic processes and cardiovascular function are examined, are: leptin, adiponectin, interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1) and tumor necrosis factor alpha (TNF-α)[1,7]. Leptin levels are elevated in obese subjects, along with TNF-α, IL-6 and PAI-1 that are proatherogenic and prodiabetic, in contrast to plasma adiponectin that protects against vascular damage and metabolic syndrome and is reduced, as it would be expected[8]. The variety in cytokine profile, along with the anatomic and cellular diversity that differentiate SCAT and VAT clarify and support the physiological and metabolic properties excreted by each adipocyte group. VAT cells allow for increased insulin-mediated glucose uptake and are more insulin-resistant and lipolysis-prone that those of SCAT. In contrast, the latter, exhibit a greater capacity for postprandial free fatty acid and triglyceride uptake and storage[1]. Taking into consideration the pivotal role of VAT in metabolic impairment, as such is supported by its aforementioned properties, it is comprehensible that studying the metabolic properties of visceral adiposity and mainly, organ-specific depositions, such as epicardial fat, has been incremental in the process of stratification of cardiometabolic risk factors. In this review, we aim to compare the morphology of epicardial fat deposits between non-diabetic individuals and subjects with type 2 diabetes mellitus (T2DM). Moreover, we will discuss the affect excreted by the antidiabetic substances in epicardial VAT, while contemplating on its clinical utility, as estimated by means of cardiovascular risk reduction.

FUNCTION AND COMPOSITION OF EPICARDIAL FAT

Epicardial adipose tissue (EAT) is an adipocyte depot of VAT with anatomical continuity to the myocardial tissue, located under the visceral layer of the pericardium[9]. It has been suggested that it can serve as a quantifiable and modifiable therapeutic target for cardiovascular adverse events, as it can be measured with non-invasive imaging techniques such as two-dimensional echocardiography, computed tomography (CT) and magnetic resonance imaging (MRI)[10]. Spatial imaging, as such is provided by MRI and CT scans, is preferable to that of two-dimensional echocardiography technique, in order to accurately measure the thickness of EAT. Along with technical shortcomings, operator- and subject- related variability deem echocardiographic imaging a formidable solution solely because of the rapid and cost-effective patient assessment it facilitates. Otherwise, MRI is considered to be the gold-standard method for EAT quantification and area placement, even though three dimensional image reconstruction by utilizing multidetector-row CT is slightly superior in achieving the latter[10,11].

On a cellular level, the epicardial adipocytes are embryologically derived from the splachnopleuric mesoderm, similarly to the mesenteric and omental adipocytes. EAT is characterized by high cellularity, defined by the concentration of adipocytes in this tissue being notably higher than that of other depots of adipose tissue[12]. EAT is a depot of white adipocytes, cells that specialize in energy storage, as opposed to brown adipocytes that are involved in energy expenditure[13].

EAT extends on an area exceeding 80% of the myocardial total surface in an otherwise healthy individual, spreading heterogeneously, mostly accumulating on the lateral and anterior walls of the right atrium[14]. The physiological structure and composition of EAT variates depending on age, gender, body weight and ethnicity[14]. The properties of EAT and its contribution in physiological and pathophysiological pathways have been extensively described. Due to its spatial distribution, EAT acts as a mechanical and thermoprotective layer for the myocardial tissue and coronary arteries. Through endocrine and paracrine function, epicardial adipocytes
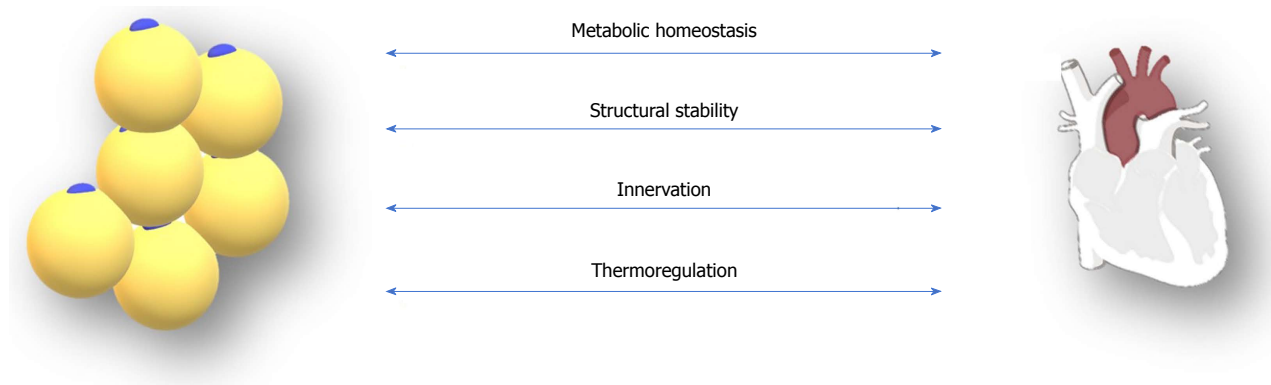
Figure 1 Mechanisms involved in the crossplay between the heart and the epicardial adipocytes.

ameliorate endothelial response of the coronaries and insulin sensitivity, while reducing oxidative stress of the cardiac tissue. Additionally, the small adipocytes of EAT are characterized by high rates of free fatty acids turnover, allowing for both energy supply and storage as demand shifts[14].

EPICARDIAL FAT, TYPE 2 DIABETES MELLITUS AND CORONARY ARTERY DISEASE

The prevalence of type 2 diabetes mellitus (T2DM) has quadrupled in the last three decades according to International Diabetes Federation (IDF) reports. The epidemic escalation has been attributed to numerous factors including population aging as a result of improved healthcare, socioeconomic development, unhealthy diet regimes and sedentary lifestyle[15].

EAT has been associated with numerous pathophysiological processes, such as coronary artery disease[16-18], even though the significance of such association has not been adequately supported by all relevant studies[19-20], electrophysiological abnormalities of the heart[21,22], cardiovascular disease in human immunodeficiency virus treated with antiretroviral therapy[23], amplified severity of non-alcoholic fatty liver disease[24,25], metabolic syndrome[26-29] and increased cardiovascular risk along with decline of renal function in individuals with T2DM[30-34].

The pathophysiological pathways linking T2DM and EAT, support a multifactorial causative relationship between EAT attributes and structure such as volume and endocrine function and cardiovascular disease severity in the diabetic individual.

EAT deposition can be associated with coronary vascular disease pathogenesis mainly by the dysregulation of cardiac metabolic processes and the disruption of the epicardial and myocardial structural integrity. Other mechanisms that could be involved in the interaction between EAT and coronary vasculature are nerve damage and impaired cryoprotection of the heart[35,36]. Furthermore, the epicardial adipocytes exhibit and arrhythmogenic potential, a theory suggested by many clinical trials ex-

ploring the causative relationship between EAT and atrial fibrillation[21] (Figure 1).

EPICARDIAL FAT AND ANTIDIABETIC DRUGS

Biguanides

Metformin is the most common first-line treatment choice for T2DM and a member of the biguanides drug class. Oral administration of the substance affects the liver and gut metabolic pathways in order for its hypoglycemic attributes to be put into effect[37]. Hepatic gluconeogenesis, glucose uptake, glycolysis and glucogen synthesis are some of the processes altered by metformin *via* AMP-activated protein kinase (AMPK)-dependent and -independent pathways[38].

At this point in time, there seem to be no randomized controlled trials designed for clarification of the effects excreted by metformin on the volume or function of EAT. Despite the fact that metformin has not been compared with placebo, as of yet, studies conducted on sitagliptin and liraglutide as add-on therapy to metformin monotherapy, combined with epicardial fat measurement, can be used as a preliminary source of data[39,40].

Results from these trials confirm the inferiority of metformin monotherapy when compared to metformin/sitagliptin and metformin/liraglutide for reduction of EAT volume. The findings can be either attributed to the synergy of two antidiabetic substances, affecting the EAT in a more effective manner than metformin alone, or to the complete lack of action of the biguanide class on the cardiac VAT deposits. The latter is supported by the results of the study performed by Iacobellis *et al*[40], that noted no EAT reduction in the metformin group during the 6-mo follow up period. Conversely, metformin has been previously shown to have positive effects on VAT, inducing its reduction on diabetic subjects[41]. Furthermore, studies have confirmed a metformin-induced increase of plasma omentin-1 levels, an adipokine produced by epicardial fat that ameliorates insulin sensitivity, inflammatory response and cardiovascular function[42]. Given the contradicting evidence concerning metformin, there is need for further research, as a definite conclusion on the manner by which biguanides interact with epicardial fat can only be provided

by a randomized controlled trial with EAT measurement.

Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors (α-GIs) are a class of anti-diabetic drugs acting in the epithelium of the small intestine mainly by delaying the digestion of carbohydrates through reversible and competitive inhibition of intestinal alpha-glucosidases, consequently reducing glucose absorption and attenuating postprandial hyperglycemia[43]. Some α-GIs are acarbose, miglitol and voglibose. Similarly to the biguanide class of antidiabetic medication, there is a lack of data concerning the administration of α-GIs and their effect on EAT mass, volume or metabolic activity.

Thiazolidinediones

Thiazolidinediones (TZDs), also known as glitazones, are peroxisome proliferator-activated receptor (PPAR) agonists with numerous actions, spanning from glycemic and lipid control to inflammatory signaling and cell cycle mediation[44]. The phenomenon of glitazone treatment and subsequent increase in body weight that has been supported by the results of numerous studies appears to be tissue-specific, since the VAT depot of the subjects remains unaffected while there is a shift of excess energy storage towards the SCAT[45-47].

Furthermore, pioglitazone treatment in T2DM or metabolic syndrome has been shown to attenuate the inflammatory signature of EAT by means of decreased expression of proinflammatory interleukins (IL) such as IL-1β, IL-1Ra and IL-10[48]. In addition to the positive effect on the metabolic profile of EAT, pioglitazone can affect the epicardial fat depot directly. Nagai *et al*[49] recruited 97 T2DM individuals that were divided into two groups according to baseline EAT thickness and underwent therapy with pioglitazone, along with EAT thickness measurement, at the beginning and after a nine-month follow-up period. Pioglitazone reduced the EAT thickness in both groups, with more prominent results in the subjects that had a greater EAT depot at baseline.

A different TZD, rosiglitazone, when administered to mice, induced the expression of brown adipose tissue-specific proteins by the EAT, a tissue type normally presenting having a hormonal profile consistent with that of white adipose tissue[50]. Brown adipose tissue has been linked to high rates of lipid turnover and reduced body weight, while it is essential for thermogenesis and homeostasis, in contrast to white adipose tissue that serves as an energy reservoir for the body[51].

The data derived from the studies examining the effect of glitazones and EAT correlates with the established theory that TZD-induced weight gain is not concurrent with VAT deposition. Moreover, TZDs appear to have a favorable effect on EAT both by regulation of endocrine functions and mass reduction.

Incretins

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that delays gastric motility, supresses appetite, stimulates glucose-dependent insulin and decreases glucagon secretion[52]. The enzyme dipeptidyl peptidase-4 (DPP-4) deactivates GLP-1 interrupting all incretin-stimulated signalling. DPP-4 inhibitors (DPP-4i) are one of the two categories of antidiabetic drugs acting on the incretin pathway, the other being GLP-1 receptor agonists (GLP-1 RA)[52]. DPP-4i inhibit both GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) degradation, thereby increasing plasma concentrations and stimulating the pancreatic β-cell in order to better regulate glucose homeostasis.

DPP-4 inhibitors

The class of DPP-4is includes sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin[53]. Sitagliptin is the only DPP-4i whose effect on epicardial fat has been studied at this point in time. Lima-Martínez *et al*[39] formed a 24-wk interventional plan for 26 obese subjects with T2DM inadequately controlled on metformin monotherapy. Subjects meeting the inclusion criteria were introduced to a new regimen, receiving sitagliptin/metformin at a dosage of 50 mg/1000 mg respectively, twice a day. EAT deposits were reduced in size by approximately 15% (from 9.98 ± 2.63 to 8.10 ± 2.11 mm, $P = 0.001$) while the percentage of reduction in EAT was analogous to that of VAT (r = 0.456, $P = 0.01$).

While the aforementioned study establishes a favourable effect of sitagliptin on the mass of epicardial VAT, there is definite need for further research, in order to establish the reduction of EAT as a class effect of DPP-4is[39].

GLP-1 receptor agonists

GLP-1 RAs utilize the "incretin effect", similarly to DPP-4 inhibitors, so as to attenuate the diabetes-induced hyperglycemia. GLP-1 RAs are divided into short- and long-acting compounds that activate the GLP-1 receptor in a manner similar to that of the endogenous GLP-1[54]. Epicardial adipocytes have been shown to express GLP-1 and 2 receptor genes by use of RNA sequencing, while the possible quantity and dispersion pattern of the receptors in vivo has not been described[55]. Furthermore, GLP-1 and GLP-1 receptor signaling affect the differentiation and growth of adipocytes by regulation of fatty acid synthase activity[56]. Even though, the effects of numerous GLP-1 RAs have been studied in correlation to the metabolic regulation or mass reduction of visceral adipose tissue, the clinical trials concerning organ-specific deposits are few[40,57-60]. Current data on EAT remodeling by GLP-1 RAs is derived by two studies, conducted with liraglutide and exenatide[40,60].

A trial designed by Iacobellis *et al*[40] included 95 T2DM obese subjects with hemoglobin A1c $\leqslant$ 8% while being treated with metformin. The patients were randomized into two groups to either receive a combination of metformin/liraglutide, with the latter being administered once daily, in doses up to 1.8 mg, or stay on metformin monotherapy, up to 1000 mg administered twice daily

for 6 mo. EAT thickness measurements were acquired by ultrasound imaging at baseline and at 3 and 6 mo. Subjects in the liraglutide group presented with a decline in EAT thickness, 29% and 36% reduction from baseline at 3 and 6 mo respectively. Given that there were no similar changes in the metformin group, the EAT mass reduction is considered to be an effect of the liraglutide treatment, or possibly a result of the synergy between the two antidiabetic substances.

The study involving exenatide had a broader spectrum than that of liraglutide, examining the effect of the GLP-1RA on numerous VAT depots including epicardial, myocardial, hepatic and pancreatic adipose pads. Measurements of EAT thickness were performed by magnetic resonance imaging and spectroscopy at baseline and at 26 wk. A total of 44 obese individuals with uncontrolled T2DM, originally receiving oral therapy, were randomized to two groups, either receiving exenatide or other treatment chosen according to the local guidelines. EAT was reduced by approximately 8.8% after treatment with exenatide and by 1.2% on the patients receiving oral therapy, with the difference between the two being statistically significant ($P = 0.003$)[60].

Current research conducted on incretin treatment and ectopic adipose tissue deposition supports the theory that EAT reduction could be a class effect of GLP-1RAs and possibly a mediator of their beneficial actions on cardiovascular disease in the diabetic and obese subjects.

SGLT-2 inhibitors

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a novel class of antidiabetic substances that bind on the SGLT2 transporter in the proximal tubule of the kidney, facilitating glucose excretion *via* hindering reabsorption. SGLT2-mediated reabsorption constitutes the main pathway by which the renal system maintains glucose homeostasis[61]. Administration of SGLT2 inhibitors in obese individuals with T2DM has been linked with abdominal VAT size reduction[62]. Additionally, the effects of SGLT2 inhibition on tissue-specific depots such as EAT have been clarified by studies performed on luseogliflozin, ipragliflozin, canagliflozin and dapagliflozin[63-67].

EAT measurements following a 12-wk period of luseogliflozin administration demonstrate that treatment with luseogliflozin can reduce EAT volume in combination with adipocyte-related inflammation and metabolic dysregulation on type 2 diabetic patients. Along with EAT, numerous parameters were modified after luseogliflozin therapy including body weight, fasting plasma glucose, insulin resistance and C-reactive protein (CRP) levels. A positive correlation was established between CRP and EAT reduction (r = 0.493, $P = 0.019$), suggesting a concurrent effect of the SGLT2 inhibitor on both the adipose tissue mass and metabolic activity[63].

Similar results concerning both EAT and biomarkers reduction were acquired after ipragliflozin administration, in a study designed similarly to that conducted for luseogliflozin. The two models differed in the selection of the study population, with luseogliflozin treatment being applied to obese subjects while ipragliflozin was administered to non-obese T2DM individuals[64].

Yagi *et al*[65] studied the interaction of canagliflozin and EAT during a 6-mo period of treatment. The sample consisted of type 2 diabetic individuals, each of which was administered 100 mg of canagliflozin once daily. During the follow-up period EAT was evaluated by echocardiographic imaging while VAT and SCAT size fluctuation was monitored by use of impedance methods. The mean EAT thickness values were 9.3 mm and 7.3 mm at baseline and at 6 mo, respectively, with the change observed being statistically significant ($P < 0.001$) while there was only a trend for VAT and SCAT reduction.

Dapagliflozin and epicardial adiposity were examined through two different clinical trials, studying both the shift in metabolic activity and size of the adipocytes after treatment[66,67]. The metabolic profile of adipocytes promoted by dapagliflozin was assessed *ex vivo* on fat explants obtained from patients undergoing cardiac surgery on a trial designed by Díaz-Rodríguez *et al*[66]. Glucose uptake, transporter expression and adipokine secretion patterns were altered as a result of dapagliflozin application, a change indicative of a positive metabolic reform of the tissue induced by SGLT2 inhibition. Simultaneously, Sato *et al*[67] followed a more conventional approach, estimating the dapagliflozin-induced EAT volume reduction, by means of computed tomography imaging. Individuals receiving both dapagliflozin and other regimens for T2DM control were observed for 6 mo, with biomarker and EAT measurement at baseline and following completion of the study. While the two groups had similar EAT size measurement before the initiation of dapagliflozin therapy, the patients receiving the SGLT2 inhibitor presented with a greater reduction of epicardial VAT volume after treatment (-16.4 ± 8.3 for the dapagliflozin *vs* 4.7 ± 8.8 cm^3 for the control group, $P = 0.01$), combined with lowered plasma levels of inflammatory adipokines.

Numerous studies conducted on many members of the SGLT2 inhibitor class of antidiabetic substances support the conclusion that EAT undergoes a multifaceted remodelling after SGLT2 inhibition, a trend that could be considered a class effect. The interconnection established between SGLT2 inhibitors and a known factor of cardiovascular risk such as epicardial adiposity could elucidate the manner by which the members of this class are cardioprotective, while, providing grounds for further therapeutic targeting of EAT (Figure 2).

CONCLUSION

Epicardial adipose tissue exhibits a unique metabolic and pathophysiologic profile, as a result of its anatomical location and its cellular composition, rendering it an appealing therapeutic target for reducing cardiovascular risk and enabling endocrine homeostasis in the dysmetabolic individual. The recent studies concerning the effect of the antidiabetic substances on the multifactorial

Biguanides	No effect/Possible synergistic effect with DPP-4 and/or GLP-1[39, 40]
Alpha-Glucosidase Inhibitors	Lack of data concerning the effect of this class
Thiazolidinediones	Decreased inflammatory cytokine release and thickness of EAT (pioglitazone) modulation of cellular hormonal profile (rosiglitazone)[49, 50]
Dipeptidyl peptidase-4 inhibitors	Reduction of EAT thickness (sitagliptin)[39]
Glucagon-like peptide-1 receptor agonists	Reduction of EAT thickness (liraglutide and exenatide)[40, 60]
Sodium-glucose cotransporter 2 inhibitors	Reduction of EAT thickness (luseogliflozin, ipragliflozin, canagliflozin, dapagliflozin) and inflammation (luseogliflozin, ipragliflozin, dapagliflozin)[63-67]

Figure 2 Antidiabetic drug and their effect on epicardial adipose tissue.

cardiomyopathy of the diabetic patient and, by extension, on epicardial adiposity, have yielded interesting results that support the use of treatment for a targeted approach, in order to reduce the size and metabolic activity of ectopic adipose tissue clusters. Despite the capacity of certain treatment regimens, mostly newer agents like GLP-1 agonists and SGLT-2 inhibitors, in the manipulation of both structural and functional parameters of the epicardial adipose tissue, the clinical efficacy of this approach remains unsubstantiated for the time being. There is definite need for further research, in order to elucidate whether the targeting of epicardial adiposity facilitates the procurement of better outcomes for individuals with diabetes and cardiovascular disease, while, additionally, clarify the manner by which the antidiabetic substances can attain such results.

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Basic Study

Effects of glucagon-like peptide 1 analogs in combination with insulin on myocardial infarct size in rats with type 2 diabetes mellitus

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Abstract

AIM
To evaluate the effects of glucagon-like peptide-1 analogs (GLP-1a) combined with insulin on myocardial ischemia-reperfusion injury in diabetic rats.

METHODS
Type 2 diabetes mellitus (T2DM) was induced in male

Wistar rats with streptozotocin (65 mg/kg) and verified using an oral glucose tolerance test. After anesthesia, the left coronary artery was occluded for 40 min followed by 80 min reperfusion. Blood glucose level was measured during surgery. Rats were randomized into six groups as follows: (1) control rats; (2) insulin (0.1 U/kg) treated rats prior to ischemia; (3) insulin (0.1 U/kg) treated rats at reperfusion; (4) GLP-1a (140 mg/kg) treated rats prior to ischemia; (5) GLP-1a (140 mg/kg) treated rats at reperfusion; and (6) rats treated with GLP-1a (140 mg/kg) prior to ischemia plus insulin (0.1 U/kg) at reperfusion. Myocardial area at risk and infarct size was measured planimetrically using Evans blue and triphenyltetrazolium chloride staining, respectively.

RESULTS
There was no significant difference in the myocardial area at risk among groups. Insulin treatment before ischemia resulted in a significant increase in infarct size (34.7% ± 3.4% *vs* 18.6% ± 3.1% in the control rats, $P < 0.05$). Post-ischemic administration of insulin or GLP-1a had no effect on infarct size. However, pre-ischemic administration of GLP-1a reduced infarct size to 12% ± 2.2% ($P < 0.05$). The maximal infarct size reduction was observed in the group treated with GLP-1a prior to ischemia and insulin at reperfusion (8% ± 1.6%, $P < 0.05$ *vs* the control and GLP-1a alone treated groups).

CONCLUSION
GLP-1a pre-administration results in myocardial infarct size reduction in rats with T2DM. These effects are maximal in rats treated with GLP-1a pre-ischemia plus insulin at reperfusion.

Key words: Glucagon-like peptide-1 analog; Insulin; Myocardial ischemia-reperfusion injury; Infarct size; Type 2 diabetes mellitus; Rats; Experimental research

Core tip: In addition to their glucose-lowering effects, glucagon-like peptide-1 analogs (GLP-1a) were shown to exhibit cardioprotective effects. However, the optimal protocol of GLP-1a administration for infarct size reduction has not been determined yet. Additionally, it is important to investigate the effects of GLP-1a combined with other antidiabetic drugs on myocardial infarct size. Thus, we evaluated the effects of GLP-1a with and without insulin on infarct size in rats with type 2 diabetes mellitus. We found that GLP-1a administration prior to ischemia resulted in significant infarct size reduction. Infarct size reduction was maximal in rats treated with GLP-1a before ischemia plus insulin at reperfusion.

Zykov VA, Tuchina TP, Lebedev DA, Krylova IB, Babenko AY, Kuleshova EV, Grineva EN, Bayramov AA, Galagudza MM. Effects of glucagon-like peptide 1 analogs in combination with insulin on myocardial infarct size in rats with type 2 diabetes mellitus. *World J Diabetes* 2018; 9(9): 149-156 Available from:

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is considered a risk factor for cardiovascular diseases with an approximately three-fold increased risk of myocardial infarction (MI). Normalizing glucose variability can prevent future cardiovascular complications. Safe blood glucose levels during MI (10 mmol/L, ideally < 8.7 mmol/L, not below 4.5-5 mmol/L) are very difficult to achieve using insulin monotherapy, which is the main therapy option in the acute period of MI[1-3].

Therefore, it is necessary to find other therapeutic options to control blood glucose levels during MI. A possible candidate is glucagon-like peptide-1 analogs (GLP-1a) due to their high efficiency and low risk for hypoglycemia[4,5]. Moreover, accumulated data has shown that GLP-1a exhibits independent positive pleiotropic effects on the cardiovascular system[5-13]. However, only few studies investigated the use of GLP-1 during acute MI in patients with T2DM. The most convenient way to study the pleiotropic effects of new antidiabetic drugs involves the use of experimental models[14].

Recent studies have indicated that GLP-1a can exert beneficial effects on the cardiovascular system. The mechanisms underlying these positive effects included both indirect effects on insulin secretion, glucose uptake, and free fatty acid metabolism in the peripheral and central nervous systems, and direct effects on GLP-1 receptors in the myocardium. Although there are numerous studies on the cardiovascular effects of GLP-1a, most studies use recombinant GLP-1 infusion that is not used in clinical practice. In addition, emphasis was placed on monotherapy with GLP-1a[10]. Therefore, it is of great interest to investigate the effects of the clinically used GLP-1a or GLP-1 mimetics on the cardiovascular system and evaluate their cardioprotective effects in combination with insulin therapy, which is more clinically applicable. In addition, it is important to study the effects of GLP-1a administration at different stages of the experiment (before and during MI) to determine the appropriate dosage regimen in patients.

Therefore, in this study, we aimed to investigate the effects of GLP-1a combined with insulin on blood glucose levels, severity of myocardial damage, and mortality in experimental MI in rats with streptozotocin-induced T2DM.

MATERIALS AND METHODS

Animals
Seventy male Wistar rats were used in this study. Both neonatal STZ-induced T2DM and MI were induced in these rats. Experimental studies were conducted at the Federal State Budget Scientific Institution "Institute of Experimental Medicine" in cooperation with the staff of the

Laboratory of Chemistry and Pharmacology of Medicine, in accordance with the "Guidelines for the Care and Use of Laboratory Animals" and "A guide to experimental (preclinical) study of new pharmacological substances," with observance of the principles of humanity, European Directives (86/609/EEC), and Helsinki Declaration.

T2DM model
We used the streptozotocin(STZ)-nicotinamide model of diabetes. Induction of diabetes in rats was carried out by a single intraperitoneal injection of STZ at 65 mg/kg, dissolved in citrate buffer (pH 5.5). For selection of rats to be used in the study, blood glucose level was measured at the age of 3 mo, followed by an oral glucose tolerance test after administration of 40 % w/v glucose solution at a dose of 3 g/kg. Diagnostic criteria for T2DM included fasting blood glucose levels from 7 to 14 mmol/L (OneTouch Select glucometer, LifeScan Inc., Milpitas, CA, United States) and two-fold increase in the area under the glucose curve of the oral glucose tolerance test, compared with that in the control group[15,16].

Myocardial ischemia-reperfusion model
Rats were anesthetized using chloral hydrate solution (400 mg/kg), tracheotomized, and ventilated (SAR-830P, Stoelting, United States) using room air, with a tidal volume of 2 mL/100 g and a rate of approximately 60 breaths/min. The core body temperature was maintained at $37 \pm 0.5\,^{\circ}C$ using a feedback-controlled heating pad (TCAT-2LV controller, Physiotemp Instruments Inc., Clifton, NJ, United States). The left carotid artery and right femoral vein were cannulated for measurement of the mean arterial pressure (MAP) and maintenance of anesthesia, respectively. Lead Ⅱ of the electrocardiograph was monitored for determination of the heart rate (HR) and arrhythmias. After a 10 min stabilization, a left thoracotomy was performed. A 6-0 polypropylene thread was placed around a prominent branch of the left coronary artery, and the ends were passed through a polyethylene tube as an occluder. Exclusion criteria were MAP < 50 mmHg and/or HR < 300 bpm at any time point during the experiment[17].

Experimental protocol
Reperfusion was started 40 min after the onset of ischemia by removing the ligature from the coronary artery. After another 80 min, the ischemic lesion was assessed. Figure 1 shows the experimental protocol. Animals were randomly divided into six groups, as follows: Group 1: control rats with T2DM without therapy; Group 2: rats treated with insulin prior to MI (IpMI) at a dose of 0.1 U/kg 1.5 hr before induction of AMI; Group 3: rats treated with insulin after MI (IaMI) at a dose of 0.1 U/kg 40 min after coronary artery ligation; Group 4: rats treated with GLP-1a prior to MI (GLP1pMI) at a dose of 140 mg/kg 1.5 hr before ischemia; Group 5: rats treated with GLP-1a after MI (GLP1aMI) at a dose of 140 mg/kg 40 min after ischemia; Group 6: GLP1pMI + IaMI at a GLP-1a dose of

140 mg/kg 1.5 hr before ischemia and at an insulin dose of 0.1 U/kg 40 min after ischemia.

Infarct size measurement
At the end of the experiment, the left coronary artery was re-occluded, followed by administration of 0.5 mL of 5% Evans blue (MP Biomedicals, Solon, OH, United States) *via* the femoral vein for measurement of the area at risk (AR). The hearts were excised and cut into five 2 mm thick slices parallel to the atrioventricular groove. The basal surface of each slice was digitally photographed. The slices were immersed in 1% solution of 2,3,5-triphenyltetrazolium chloride (MP Biomedicals, Solon, OH, United States) at 37 ℃ (pH 7.4) for 15 min and photographed again for determination of infarct area (IA). The images were digitized using Adobe Photoshop CS. The AR was expressed as a percentage of the whole slice, and the IA was expressed as a percentage of AR. Values of AR and IA for each heart were obtained by calculating mean values of the slices. Rats with AR 15% were excluded from the study. Infarct size measurement and data analyses were performed by an investigator blinded to the study groups.

Evaluation of blood glucose levels
In addition, blood glucose levels were monitored during the experiment. A blood glucose test was performed prior to T2DM induction, after T2DM induction, a three day measurement with an interval of two to three days, during the glucose tolerance test, and thereafter every week before the operation. During the operation, blood was collected for glucose monitoring according to the following protocol: 1.5 hr before MI induction, immediately before MI, and then every 20 min. Measurement of blood glucose levels was performed at all points with the Accu-Chek glucometer using diagnostic test strips[3,18-20].

Statistical analysis
Statistical analyses were carried out using the IBM SPSS Statistics 23 program (SPSS Inc., Chicago, IL, United States) and were performed by a biomedical statistician. Data were presented as the mean ± SD. To evaluate the differences between dependent samples, the non-parametric Wilcoxon test was used, whereas the Mann-Whitney test was used to evaluate the reliability of the differences between independent variables. *P* values < 0.05 were considered statistically significant.

RESULTS
Blood glucose levels and glycemic variability
We assessed the features of the glycemic profiles in experimental animals. Data on glycemic variability and number of episodes of hypoglycemia in rats are presented in Table 1. The highest glycemic variability was observed in the rats treated with insulin monotherapy, whereas the lowest glycemic variability was achieved in the rats receiving GLP-1a (*P* < 0.05). It is noteworthy that glycemic variability in the rats treated with combined GLP-1a and

Table 1 Number of hypoglycemia episodes and glycemic variability in experimental animals						
Group	**Control**	**IpMI**	**IaMI**	**GLP1pMI**	**GLP1aMI**	**GLP1pMI + IaMI**
Hypoglycemia episodes (*n*)	0	5	3	1	2	1
Glycemic variability	4.48 ± 0.74	6.29 ± 0.9[a]	5.57 ± 1.3	3.79 ± 0.65[a]	3.60 ± 0.65[a]	3.00 ± 0.42[a]

[a]$P < 0.05$, *vs* control group. Control: Rats with T2DM without therapy; IpMI: Rats treated with insulin prior to MI; IaMI: Rats treated with insulin after MI; GLP1pMI: Rats treated with GLP-1a prior to MI; GLP1aMI: Rats treated with GLP-1a after MI; GLP1pMI + IaMI: Rats treated with GLP-1a prior to MI and with insulin after MI.
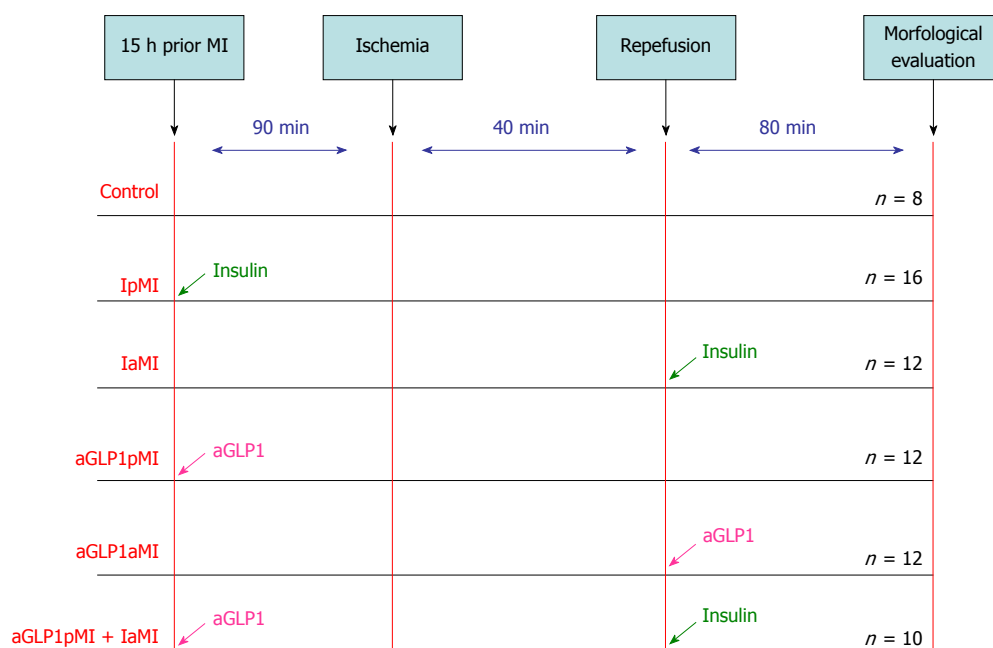

Figure 1 The experimental protocol. Group 1: Control rats with T2DM without therapy; Group 2: IpMI (rats treated with insulin prior to MI); Group 3: IaMI (rats treated with insulin after MI); Group 4: GLP1pMI (rats treated with GLP-1a prior to MI); Group 5: GLP1aMI (rats treated with GLP-1a after MI); Group 6: GLP1pMI + IaMI (rats treated with GLP-1a prior to MI and with insulin after MI).

insulin was comparable with that in the rats receiving GLP-1a monotherapy. However, it was significantly lower than that in the rats treated with insulin monotherapy and in the control group rats ($P < 0.05$). The number of episodes of hypoglycemia was also high in the groups receiving insulin monotherapy, whereas hypoglycemia was practically undetected in the groups receiving GLP-1a. Statistical analysis of hypoglycemia incidence was not performed because of the small sample size. Thus, data were expressed as absolute numbers.

Mortality
In addition, we evaluated the mortality of rats during the experiment. During the experiment, 26 rats died owing to acute heart failure and/or persistent arrhythmia (Table 1). Data on the ratios of dead and surviving rats in each group are presented in Table 1. Statistical analysis of mortality was not carried out because of the small sample size.

Area at risk and infarct size
To estimate the extent of myocardial damage, the ratio of the necrotic zone area to the ischemic zone area was calculated. The AR was expressed as a percentage of the whole slice, and the IA was expressed as a percentage of AR. Values of AR and IA for each heart were obtained by calculating the mean values of the slices[21]. Results are shown in Table 2.

Figures 2 and 3 show the risk and necrotic zones. The AR did not significantly differ among groups. However, the largest zone of myocardial necrosis in relation to the ischemic zone was observed in the rats receiving insulin monotherapy before induction of ischemia.

Insulin treatment before ischemia resulted in a significant increase in infarct size (34.7% ± 3.4% *vs* 18.6% ± 3.1% in the control $P < 0.05$). Post-ischemic administration of insulin or GLP-1a had no effect on infarct size. Pre-ischemic administration of GLP-1a reduced infarct size to 12% ± 2.2%. The maximal infarct size reduction was observed in the rats treated with GLP-1a pre-ischemia and insulin at reperfusion (8% ± 1.6%, $P < 0.05$ *vs* the control and GLP-1a alone-treated groups).

DISCUSSION

The results of this experimental study confirmed the cardioprotective effects of GLP-1a, which were reported

Group	1 Control (*n* = 8)	2 IpMI (*n* = 16)	3 IaMI (*n* = 12)	4 GLP1pMI (*n* = 12)	5 GLP1aMI (*n* = 12)	6 GLP1pMI + IaMI (*n* = 10)
Ratio of surviving to dead rats (% of deaths)	5/3 (37.5%)	8/8 (50%)	7/5 (41.6%)	8/4 (30 %)	8/4 (30 %)	8/2 (20%)

Table 2 Mortality of rats expressed as percentages with different antihyperglycemic therapies

Control: rats with T2DM without therapy; IpMI: Rats treated with insulin prior to MI; IaMI: Rats treated with insulin after MI; GLP1pMI: Rats treated with GLP-1a prior to MI; GLP1aMI: Rats treated with GLP-1a after MI; GLP1pMI + IaMI: Rats treated with GLP-1a prior to MI and with insulin after MI.

Figure 2 Comparison of the necrotic zone and zone at risk between different drugs. Control: Rats with T2DM without therapy; IpMI: Rats treated with insulin prior to MI; IaMI: Rats treated with insulin after MI.

Figure 3 Comparison of the necrotic zone and zone at risk between different drugs. GLP1pMI: Rats treated with GLP-1a prior to MI; GLP1aMI: Rats treated with GLP-1a after MI; GLP1pMI + IaMI: Rats treated with GLP-1a prior to MI and with insulin after MI.

in earlier studies. In addition, we assessed the glycemic variability since inadequate control of this index has been shown to worsen the prognosis for MI in patients with T2DM[1]. GLP-1a infusion results in a decrease in blood glucose concentration to the level of fasting glycemia. However, as soon as the level of blood glucose decreases and approaches normal values, the effects of GLP-1a on insulin secretion cease because of a feedback mechanism. In addition, GLP-1a suppresses glucagon secretion from the pancreatic α-cells by means of a glucose-dependent mechanism. Thus, the fact that GLP-1a cannot cause severe hypoglycemia is clinically important[22,23]. The results of this study suggested that administration of GLP-

1a reduced glycemic variability, regardless of the time of administration and combination with other drugs.

Previous studies have evaluated the effects of GLP-1a in the cardiovascular system[14]. In addition, one study investigated the effects of GLP-1 agonists on the endothelial function of blood vessels in patients with T2DM and stable angina pectoris[24]. GLP-1a significantly improved endothelium-dependent vasodilatation of the brachial artery in samples with acetylcholine in patients with T2DM. Moreover, previous studies showed that recombinant GLP-1 infusion improved left ventricular function in patients with T2DM and severe heart failure[25]. In addition, a significant decrease in the systolic blood

pressure was observed approximately two weeks after the initiation of therapy in LEAD studies[26]. This anti-hypertensive effect of GLP-1a might be attributed to its vasodilator effects by increasing the expression of endothelial nitric oxide synthase. Alternatively, it could result from its natriuretic or diuretic action[27].

Administration of GLP-1 agonists at high doses resulted in a decrease in the levels of three biomarkers of cardiovascular risk, including triglycerides, inhibitor of plasminogen-1 activator (PAI-1), and natriuretic peptide type B, compared to placebo. Accordingly, LEAD studies concluded that GLP-1a provided more effective target achievement of the final complex, combining three important parameters of metabolic control, HbA1c, systolic blood pressure, and body weight, compared with that of other hypoglycemic drugs. In addition, since GLP-1a results in an indirect increase in insulin secretion, it also achieves all the cardioprotective effects of insulin therapy. Thus, GLP-1a therapy has the advantage of maintaining the positive effects of insulin therapy by eliminating its potential complications.

Our study showed the cardioprotective effects of GLP-1a. We suggested that the use of both GLP-1a alone and in combination with insulin reduced the necrotic zone area. Our research protocol was as close as possible to the clinical situation and ensured optimal translation of the results of the present study to the clinic. Insulin-treated rats exhibited significant differences depending on whether insulin was administered prior to or after the induction of ischemia.

The mechanisms of insulin action in MI are now well studied. In particular, the cardioprotective effects of insulin at reperfusion are attributable to the increase in the production of phosphatidylinositol 3-kinase, which promotes the synthesis of antiapoptotic protein kinases, inhibits apoptosis, and promotes the survival of cardiomyocytes. In addition, it is known that insulin lowers the concentration of free fatty acids and ketone bodies in the myocardium, which increases the activity of pyruvate dehydrogenase to a certain extent and decreases the accumulation of lactate in the myocardium. These effects significantly improve the regulation of metabolic processes in the damaged myocardium and subsequently reduce the mortality and duration of hospitalization. Glucose uptake by the myocardium is significantly enhanced or even normalized with adequate insulin therapy. This, in turn, has a positive effect on prognosis and improves the systolic function and left ventricular ejection fraction. Additionally, insulin can suppress inflammation and enhance fibrinolysis (by decreasing the activity of antifibrinolytic factors) in patients with acute MI with ST segment elevation, receiving low-dose insulin infusion and fibrinolytic therapy. These effects of insulin, along with its vasodilator and antiplatelet actions, promote reperfusion at the level of the epicardium and microcirculatory bed, and thus protect the myocardium[28].

In addition to the above evidence, our hypothesis on the putative infarct-limiting effect of insulin pretreatment was based on the data obtained in the study of Fuglesteg *et al*[29] who showed mTOR-dependent infarct size reduction after preischemic insulin administration in the Langendorff-perfused rat heart. There are, however, studies that have not confirmed this fact. Therefore, we thought to check if insulin is really cardioprotective when administered prior to ischemia. Our results showed that insulin monotherapy resulted in high glycemic variability and low survival rate in experimental animals with acute myocardial ischemia. In the experimental group treated with insulin before induction of ischemia the necrotic area was the largest among all other groups. The lowest percentage of myocardial necrosis was observed in the rats treated with GLP-1a before induction of ischemia and in rats receiving combination therapy.

The main limitation of this study was the small sample size that did not allow full-scale statistical analysis to assess the mortality of animals during the experiment and the number of episodes of hypoglycemia. However, this sample size allowed us to fulfill the main goal of this study and draw conclusions on the effects of GLP-1a in MI.

We suggest that the pronounced positive effects of GLP-1a during the course of MI therapy occurred when it was administered at the onset of infarction. This could be explained by the fact that the required drug concentration and effects in the myocardium could only be achieved when the drug was administered prior to the induction of ischemia.

In conclusion, GLP-1a pre-ischemic administration results in myocardial infarct size reduction in rats with T2DM. These effects are maximal in rats treated with GLP-1a pre-ischemia plus insulin at reperfusion.

ARTICLE HIGHLIGHTS

Research background
Type 2 diabetes mellitus (T2DM) is associated with an increased risk of myocardial infarction (MI) and poorer prognosis. Recent studies demonstrate that glucagon-like peptide-1 analogs (GLP-1a) possess infarct-limiting effects in experimental settings. However, it is not clear whether GLP-1a have beneficial effects when combined with insulin.

Research motivation
In this study, we intended to compare the cardioprotective effects of GLP-1a therapy with and without concomitant insulin in acute MI in rats with T2DM.

Research objectives
The effects of pre- and post-ischemic GLP-1a and insulin administration on infarct size were studied in the rat model of MI. The effect of a combination of GLP-1a and insulin was assessed in a separate group.

Research methods
We induced T2DM in Wistar rats with streptozotocin at a dose of 65 mg/kg. Myocardial ischemia was induced by left coronary artery occlusion. Myocardial infarct size was determined histochemically. In addition, we analyzed the number and severity of hypoglycemia episodes in the experimental groups. Animals were treated with either GLP-1a or insulin.

Research results
Results of our study show that using GLP-1a before ischemia-reperfusion significantly reduced infarct size. The maximal infarct size reduction was observed in the group treated with GLP-1a prior to ischemia and insulin at

reperfusion.

Research conclusions
We have shown that insulin infusion before ischemia increased infarct size, while GLP-1a demonstrated cardioprotective effects. Post-ischemic administration of insulin or GLP-1a had no effect on infarct size. Thus, the regimen of GLP-1a and insulin administration is crucial for expression of their cardioprotective effect.

Research perspectives
Further studies with larger sample sizes can be conducted in order to develop a clinical trial and introduce new combinations of drugs with antidiabetic activity for MI therapy in patients with T2DM.

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ORIGINAL ARTICLE

Case Control Study

Association of *TCF7L2* mutation and atypical diabetes in a Uruguayan population

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Author contributions: Beloso C processed the samples from the atypical diabetes patients and controls, performed analysis and interpretation of the data, and participated in writing of the manuscript; Souto J contributed to laboratory processing of the samples and writing of the manuscript; Fábregat M acquired the patients' data and performed processing of the "classical" diabetes samples; Romanelli G contributed to the statistical analyses; Javiel G selected the patients for the protocol and attended to them in clinic, and made critical revisions related to the important intellectual content of the manuscript; Mimbacas A made substantial contributions to the conception and design of the study and critical revisions related to the important intellectual content of the manuscript, and gave final approval of the version of the article to be published.

Abstract

AIM

To investigate if mutations in *TCF7L2* are associated with "atypical diabetes" in the Uruguayan population.

METHODS
Healthy, nondiabetic controls (n = 133) and patients with type 2 diabetes (n = 177) were selected from among the presenting population at level-3 referral healthcare centers in Uruguay. Patients with type 2 diabetes were subgrouped according to "atypical diabetes" (n = 92) and "classical diabetes" (n = 85). Genotyping for the rs12255372 and rs7903146 single nucleotide polymorphisms (SNPs) in the *TCFTL2* gene was carried out with *Taq*Man® probes. Random samples were sequenced by Macrogen Ltd. (South Korea). Statistical analysis of the SNP data was carried out with the SNPStats online tool (http://bioinfo. iconcologia.net/SNPstats). The best inheritance model was chosen according to the lowest values of Akaike's information criterion and Bayesian information criterion. Differences between groups were determined by unpaired *t*-tests after checking the normal distribution or were converted to normalize the data. The association of SNPs was tested for matched case-control samples by using χ^2 analysis and calculation of odds ratios (ORs) with 95% confidence intervals (CIs). All statistical tests were performed using SPSS v10.0 and EpiInfo7 statistical packages. Significant statistical differences were assumed in all cases showing adjusted $P < 0.05$.

RESULTS
We genotyped two *TCF7L2* SNPs (rs7903146 and rs12255372) in a population-based sample of 310 Uruguayan subjects, including 133 healthy control subjects and 177 clinical diagnosed with type 2 diabetes. For both SNPs analyzed, the best model was the dominant type: rs12255372 = G/G *vs* G/T+T/T, OR = 0.63, 95%CI: 0.40-0.98, $P < 0.05$ and rs7903146 = C/C *vs* C/T+T/T, OR = 0.79, 95%CI: 0.41-1.55, P = 0.3. The rs12255372 SNP showed high association with the type 2 diabetes cases (OR = 1.60, 95%CI: 1.20-2.51, $P < 0.05$). However, when the type 2 diabetics group was analyzed according to the atypical and classical subgroupings, the association with diabetes existed only for rs12255372 and the classical subgroup (*vs* controls: OR = 2.1, 95%CI: 1.21-3.75, $P < 0.05$); no significant differences were found for either SNP or atypical diabetes.

CONCLUSION
This is the first time SNPs_*TCF7L2* were genotyped in a diabetic population stratified by genotype instead of phenotype. Classical and atypical patients showed statistical differences.

Key words: TCF7L2; Atypical diabetes; Type 2 diabetes; Latin America; TaqMan

Core tip: This is the first time single nucleotide polymorphisms (SNPs) of the *TCF7L2* gene were genotyped and comparatively assessed in Uruguayan type 2 diabetes patients with "atypical" and "classical" cases. The results show that these two populations are genotypically different. The only statistical association found involved one of the SNPs, rs12255372, and classical diabetes. No association was found to exist between either of the two SNPs examined (rs7903146 and rs12255372) and atypical diabetes. The findings in this study confirm the results of our previous investigations, which indicated that atypical and classical diabetes are two separate entities of the diabetes disease.

INTRODUCTION
Diabetes mellitus is a global public health problem, and the Uruguayan population poses no exception. The prevalence of diabetes in Uruguay is 8%[1], accounting for 3.3 million of the country's inhabitants[2]. Worldwide, type 2 diabetes (T2D) is the most common form, with incidence and prevalence having reached epidemic proportions.

Since 2009, our group has published on patients that were difficult to classify from a clinical point of view because they did not present a correlation between phenotype and genotype[3-5]. The current international guidelines for defining the type of diabetes present in an individual are still not sufficient to diagnose atypical diabetes. Patients with atypical diabetes do not fit exactly in any of the groups defined in the guidelines because they do not precisely follow the classical presentation and disease evolution and they show poor therapeutic response.

These patients have been treated in level-3 health-care settings. The atypical cases could be bypassed inadvertently by healthcare providers if the appropriate genetic and immunological analyses are not carried out, primarily because overweight or obese status is a gross indictor of insulin-resistance. Indeed, these visibly assessed features are the primary disorder considered in classification of type 2 diabetes in the internationally used recommendations of the American Diabetes Association (ADA)[6]. Atypical diabetes could be a confounder in latent autoimmune diabetes of adults (LADA) but the two are distinguishable according to several specific clues. LADA includes (1) patient onset at $\geqslant$ 30 years of age (we have found children and young people with atypical diabetes); (2) an absence of metabolic syndrome along with features of obesity, high blood pressure and high cholesterol levels (all of the atypical patients we have encountered have this condition); (3) uncontrolled hyperglycemia despite using oral agents; and (4) other autoimmune diseases (with clinical evidence for diagnosis) that are not necessarily present in atypical diabetes (*i.e.*, Graves' disease and anemia)[7].

For the work presented herein, we analyzed the association of transcription factor 7-like 2 (*TCF7L2*), one

of the major genes related to T2D, continuing with the genetic characterization of atypical diabetes patients. Our choice of this gene was based upon the remarkable amount of research that has been carried out to date on the genetic factors of diabetes, and from which *TCF7L2* has emerged as one of the strongest T2D susceptibility genes[8-10].

TCF7L2 is a Wnt signaling-associated transcription factor, expressed in the intestine, pancreas, others tissues and plays an important role in the β-cell proliferation and insulin secretion[11]. Publications reviewing the possible mechanisms have led to several theories on the processes by which altered *TCF7L2* production or function may cause diabetes. Among these, reduced insulinotropic effect of incretin hormones, of GLP-1 signaling in β-cells especially, impaired insulin processing or release, and decreased β-cell mass seem to be the most probable etiological mechanisms[12-15]. The fact that genes encoding Wnt signaling pathway factors are active in β-cells or the indication that they may be involved in insulin secretion supports the notion that β-cell dysfunction is a crucial final step on the path to diabetes[16].

The *TCF7L2* gene is located on chromosome 10q25 and is composed of two major domains: a catenin-binding domain (exon 1) and a central DNA-binding HMG domain (exons 10 and 11). Variations in this gene have been consistently associated with T2D in studies of different populations, namely those of Caucasian, Asian and African origin, and specifically involving two intronic single nucleotide polymorphisms (SNPs): rs12255372 (G>T) and rs7903146 (C>T)[17-25]. For rs12255372, homozygous carriers of the rare T allele produce 2.5-fold higher levels of *TCF7L2* transcript than wild-type carriers, while heterozygous carriers of both alleles produce 1.5-fold higher[26]. This SNP in particular affects diabetes through deficiency in insulin secretion, more so than through insulin resistance[14].

For rs7903146, it is more associated with capacity of insulin secretion than insulin resistance of the β-cell[27]. The allele T carriers permit the decrease of insulin secretion in postprandial state. This is an important characteristic becuse it is possible messure the cell response to glucose, aminoacid and incretins[28].

In this study, we investigated the association between the *TCF7L2* SNPs rs1255372 and rs7903146 in a control (nondiabetic) group and a case (diabetic) group consisting of patients with classical or atypical T2D in the Uruguayan population. This study represents the first time these SNPs have been investigated by stratifying the study population according to presence or absence of HLA and nonHLA susceptibility genes to T2D in patients with body mass index (BMI) $\geq$ 25 kg/m^2.

MATERIALS AND METHODS

We analyzed a total study population of 310 individuals, including T2D patients (*n* = 177) and controls (*n* = 133) that were enrolled in the study between 2004 and 2012.

Recruitment of patients was done by selecting from two referral diabetes healthcare centers in Montevideo, Uruguay, namely the Pasteur Hospital and CASMU-IAMPP.

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committees of both participant institutions (Law 18331). All cases and controls signed an informed consent form for participation in this investigation.

T2D patients
Patients were selected according to ADA recommen-dations[6]. We took into consideration another criterion, that being patients who had received a multidisciplinary care approach for their diabetes, showed good adherence to their treatment regimen (including a nutritional and physical activity plan according to their functional capabilities), and had received one or more oral antihyper-glycemic drugs.

For the stratification of T2D patients into the classical and atypical diabetes groupings, we used the same classification standards as in our previous studies[3-5]. For atypical diabetes, 92 of the patients met the following inclusion criteria: (1) BMI $\geq$ 25 kg/m^2 and categorization following the World Health Organization overweight and obesity guidelines (25-29.9 kg/m^2 and $\geq$ 30 kg/m^2 respectively); (2) having reached the education and nutrition plans' objectives as per international guidelines; (3) having presented doubts about their disease classifi-cation and/or not having reached a good therapeutic response (*i.e.*, no decrease of 1.5% in HbA1c levels shown in two consecutive measurements after 3 mo[29]); and (4) presence of autoimmune-diabetes-susceptibility HLA alleles (the HLA DQB1* 0201-0302 and DR 3-4 susceptibility alleles were considered for the Uruguayan population[30]). For classical diabetes, 85 patients fulfilled the (1) and (2) inclusion criteria listed above but did not present doubts about their diagnosis and did not show presence of autoimmune-susceptible genes.

Individuals who fit the inclusion criteria but had other metabolic disorders or were undergoing tumor processes were excluded from this study.

Control (healthy, nondiabetic) subjects
One hundred and thirty-three healthy, nondiabetic sub-jects were recruited from among blood donors at the Hemotherapy Department of Pasteur Hospital between 2014 and 2015. The blood donors presented for this service on a volunteer basis. Prior to the sample ex-traction, the attending doctors carried out an exhaustive questionnaire survey of each donor, which included information on chronic or infectious diseases and taking of any medication(s). Blood pressure was taken and weight and height were recorded in order to calculate the BMI (kg/m^2). Laboratory tests were carried out and individuals with infectious diseases such as human immunodeficiency virus and hepatitis A/B/C were excluded from enrollment. At the time of sample extraction, none of the donors

Table 1 Anthropometric characteristics of the study population		
	Healthy controls	**Type 2 diabetes cases**
n	133	177
Male/Female	76/57	87/90
Age (yr)	37.49 ± 13.04	63.05 ± 11.66
BMI (kg/m^2)	25.17 ± 5.35	31.73 ± 5.65

BMI: Body mass index; SD: Standard deviation.

had diabetes, but this did not rule out the possibility that they could have developed it in the future since it is a multifactorial disease. The majority of the population that donates blood has an average age of approximately 38 years.

Of the 300 individuals that were initially included as the control population, we selected those who had a BMI similar to that of the sample of patients with atypical diabetes in order to avoid a confounding variable. In addition, we had already shown in a previous study that this variable does not have a statistically significant difference between classic and atypical diabetes cases[5].

Genetic typification
DNA was obtained from peripheral blood using the standard phenol/chloroform technique. The rs12255372 (G>T) and rs7903146 (C>T) SNPs in the *TCFTL2* gene were genotyped using *Taq*Man® Probes for real-time PCR in the Rotor-Gene™ 6000 PCR machine (Corbett Research, Sydney, Australia). The primers and probes for SNP rs12255372 were designed with the Primer3Plus software (Boston, MA, United States) and AlleleID® software (Palo Alto, CA, United States), respectively. The primer sequences were as follows: forward, TCTGGCTTGGAAAGTGTA; reverse, GAGGCCTGAGTAATTATCAGAA. The probe sequence was as follows: FAM/HEX-CCAGGAATATCCAGGCAAGAAT[T/G]ACCA-BHQ1. The rs7903146 primers and probe were obtained from the catalog of genotyping experiments in *Taq*Man® SNP Genotyping Assays (Life Technologies™, Carlsbad, CA, United States). The primer sequences were as follows: forward, GCCTCAAAACCTAGCACAGC; reverse, GTGAAGTGCCCAAGCTTCTC. The probe sequence was as follows: VIC/HEX TAGAGAGCTAAGCACTTTTTAGATA[C/T]TATATAATTTAATTGCCGTATGAGG. In both cases, a commercial genotyping kit (Platinum® Quantitative PCR SuperMix-UDG; Invitrogen™ by Life Technologies™) was used for the genotyping procedure.

Melting curve analyses were performed using the Rotor-Gene™ 6000 software v.1.7 (build 75) and the accompanying algorithm. Random samples were sequenced by Macrogen Ltd. (Seoul, South Korea) and were aligned using MEGA4 (Molecular Evolutionary Genetics Analysis software, Tempe, AZ, United States).

Statistical analysis
The statistical analyses for the polymorphisms were done with the online tool for SNP analysis, SNPStats (https://www.snpstats.net/start.htm?http://bioinfo.iconcologia.net/SNPstats). The best inheritance model was chosen according to the lowest values of Akaike's information criterion (AIC) and Bayesian information criterion (BIC). Continuous variables were expressed as means and standard deviations. Differences between groups were determined by unpaired *t*-tests after verification of normal distribution, or converted to normalize the data.

The association of SNPs in matched case-control samples was tested using χ^2 analysis and calculation of odds ratios (ORs) with 95% confidence intervals (CIs). All tests were performed using the SPSS statistics package version 22 (IBM Corp., Armonk, NY, United States) and Epi Info™ statistics package version 7 (Atlanta, GA, United States). Significant statistical differences were assumed in all cases having adjusted $P < 0.05$.

RESULTS
We genotyped two *TCF7L2* SNPs-rs7903146 and rs12255372-in a population-based sample of 310 Uruguayan subjects, including 133 control subjects and 177 patients clinically diagnosed with T2D. The most relevant anthropometric values for the T2D and control groups are presented in Table 1. The T2D atypical and classical subgroups are presented in Table 2, showing the main clinical characteristics of each.

The best inheritance model was the dominant model for both SNPs analyzed: rs12255372= G/G *vs* G/T+T/T, AIC = 423.4, BIC = 430.8; and rs7903146 = C/C *vs* C/T+T/T, AIC = 426.4, BIC = 433.9. The rs12255372 SNP was the only variation that showed high association with the disease (Table 3). Comparative statistical analysis of the atypical and classical diabetes subgroups showed association only between the classical diabetes *versus* controls for rs12255372 (Table 4).

DISCUSSION
Diabetes mellitus is a complex disease, in which genetic and environmental factors are interweaved. After the discovery of *TCF7L2* as a key player in T2D etiology, several works in multiethnic populations identified two main SNPs in this gene, rs12255372 and rs7903146, and characterized them as the most relevantly associated to T2D[9,10,17,21]. In our previous works[3-5], we reclassified patients who are clinically diagnosed as T2D into two subgroups, representing the classical and atypical cases, as described in the Materials and Methods section. Intriguingly, these previous studies consistently found that the two case categories were different at the genetic level, involving several genes. In the current study, we amplified the genetic characterization of atypical diabetes in Uruguayans to include the analysis of two SNPs strongly related with T2D according to other populations studied and reported.

Analyzing the T2D study population in comparison to

Table 2 Clinical characteristics of the atypical and classical diabetes cases

	Atypical diabetes, *n* = 92	Classical diabetes, *n* = 85	*P*
Age (yr)	61.29 ± 13.08	65.74 ± 9.93	0.011[b]
Age (yr)	43.36 ± 12.62	45.93 ± 14.67	0.304
BMI (kg/m²)	32.22 ± 5.48	31.32 ± 5.96	0.288
HbA1c, %	8.27 ± 1.80	8.27 ± 1.77	0.993
Total cholesterol (mmol/L)	5.18 ± 1.14	5.53 ± 1.16	0.044[a]
HDL (mmol/L)	1.24 ± 0.29	1.32 ± 0.33	0.080
LDL (mmol/L)	2.84 ± 1.02	3.26 ± 1.11	0.010[b]
Triglycerides (mmol/L)	2.24 ± 1.40	2.24 ± 1.4	0.991
TG/HDL	4.47 ± 3.41	4.43 ± 4.73	0.941

[a]$P < 0.05$, [b]$P < 0.01$, for all parameters. BMI: Body mass index; HbA1c: Glycated hemoglobin; HDL: High-density lipoprotein-cholesterol; LDL: Low-density lipoprotein-cholesterol; TG/HDL: Insulin resistance index.

Table 3 Genotype frequencies of rs12255372 and rs7903146 in controls and cases

SNPs	Healthy controls, %	Type 2 diabetes cases, %	OR (95%CI)	*P*
rs12255372 G > T				
G/T + T/T	48.9	60.5	1.6 (1.02-2.51)	0.04
G/G	51.1	39.5		
T allele	30	37	1.37 (0.97-1.92)	NS
G allele	70	63		
rs7903146 C > T				
C/T + T/T	46.6	52.5	1.27 (0.81-1.99)	NS
C/C	53.4	47.5		
T allele	29	34	1.22 (0.87-1.72)	NS
C allele	71	66		

CI: Confidence interval; NS: Non-significant; OR: Odds ratio; SNP: Single nucleotide polymorphism.

Table 4 Genotype frequencies of rs12255372 and rs7903146 in controls, atypical diabetes and classical diabetes patients

SNPs	Controls, %	Atypical diabetes, %	Classical diabetes, %	OR (95%CI)	*P*
rs12255372 G > T					
G/T+T/T	65	-	57	2.1 (1.21-3.75)	0.008
G/G	68	-	28		
G/T+T/T	65	50	-	1.2 (0.73-2.12)	NS
G/G	68	42	-		
rs7903146 C > T					
C/T + T/T	62	-	47	1.4 (0.82-2.45)	NS
C/C	71	-	38		
C/T+T/T	62	46	-	1.2 (0.67-1.95)	NS
C/C	71	46	-		

SNP: Single nucleotide polymorphism; CI: Confidence interval; NS: Non-significant; OR: Odds ratio; SNP: Single nucleotide polymorphism.

the control population showed us a significant association of the rs12255372 SNP. The same results have been found in other studies of different populations, and the presence of the T allele was also found to be associated to major proneness to T2D[21,31,32]. Subsequent analysis of our results from the T2D patients upon subgrouping according to atypical and classical cases, and in comparison with controls, showed an increase in OR when we removed the atypical patients from the analysis for rs12255372. This finding could indicate that the atypical subpopulation of T2D patients could serve as a confounding factor in general analyses of T2D patients, highlighting the potential of the overall T2D population being a mixture of case subgroups. This subgroup profile may help to explain

previous results observed in different studies that used the T2D pooled population as a unique group.

The ideas expressed above are in accordance with the notion that atypical patients could be framed as a separated group from patients with classical T2D[3]. Another perspective sets atypical diabetes in a mid-course status between type 1 diabetes and T2D, as described by Pozzilli *et al*[33]; as such, the atypical diabetes case would be located in the middle of the T2D disease spectrum. This concept goes along with the so-called "accelerator hypothesis", which states that β-cell loss could be variably accelerated by the conjunction and different weight of three different processes: insulin resistance, autoimmunity and constitution[34,35]. The β-cells of those

individuals carrying the *TCF7L2* gene mutations are more susceptible than others to the metabolic demands of insulin resistance[36], but not as susceptible as those carrying the HLA DR3/DR4 haplotype, as in the case of the atypical diabetes population, providing a combination of unfavorable genetic background, impairment in β-cell secretion and a diminished survival upon challenge with hyperglycemic stress, as well as establishing an auto-immune cell environment[14,26,37].

Interestingly, the rs7903146 SNP did not show significant association with T2D in our analyses. The Uruguayan population has a three-hybrid admixed origin-European, African and Amerindian; the Caucasian component represents a major proportion, but there is a significant mixegenation degree and a noteworthy Amerindian component of maternal origin[38]. Studies performed in different Asian populations[39-42] have found no significant association between this SNP of the *TCF7L2* gene and T2D. Thus, one could ponder the idea that the Uruguayan population may be more related to Asian populations than expected; this idea might also be supported by the theory that the American continent was populated by Asian ancestors[38].

Beyond the ethnic influence that has also been found in other studies of rs12255372 and rs7903146[17-25], this study showed that the association of rs12255372 with diabetes was increased when the population was reclassified as subgroups of case types and only the classical T2D patients were compared with controls. This finding suggests the importance of taking into consideration the existence of an atypical group, which could serve to obscure the real association of SNPs in the *TCF7L2* gene. On the other hand, it is important to note that studies using populations of patients with LADA have found differences in the polymorphisms of the *TCF7L2* gene as well as with T2D[43,44]. This finding reinforces the theory that LADA and atypical diabetes are distinct entities.

To continue the characterization of the atypical diabetes subpopulation it will be important to obtain measurements of C-peptide from the patients, so as to study if there is any difference for this marker between the subpopulations classified. The C-peptide is a precursor of insulin whose measurement shows the reserve of secretion of the same by the pancreatic β-cell. It is very useful in those patients with poor response to antihyperglycemic medication, as is the case of our atypical patients. Therefore, this technique would represent another resource to help in the classification and a more appropriate therapeutic in this population of complex patients.

Today, in our country, the investigation of C-peptide is not routinely performed and is only carried out in some specialized centers. Therefore, it becomes of paramount importance to implement the C-peptide measurement in the Healthcare Centers and Hospitals in our country. In addition, it will be important to continue characterizing the atypical diabetes subpopulation through the study of genetic markers. Identification and characterization of disease-specific genetic markers will help doctors to more readily and more accurately classify these cases, according to an etiopathogenic base. Such could also lead us to designing and implementing a therapy that will avoid or minimize trial and error time.

At the same time, therapeutic inertia would facilitate advancement of the chronic complications of this pathology. In the group of patients investigated in this study, we took into account the presence of clinical biomarkers. Although we cannot speak from a statistical point of view (due to the short time elapsed during the study period), we have managed to individualize the therapy (data not shown). In turn, this has led our patients with atypical presentation to have greater confidence in the treatment used, such as the acceptance of a timely insulinization.

Ultimately, this study showed that the application of a translational medicine research approach provides knowledge of basic science that can be applied directly in the clinic towards the resolution of complex clinical cases.

We have studied two of the most relevant SNP variants related to T2D, in the *TCF7L2* gene, in a Uruguayan diabetic population stratified by genotype differences. The present and previous works support the idea that the combined effect of several predisposition variants would turn the atypical subpopulation into a new classification and serve as therapeutic targets[45].

Currently, there are different classifications that encompass atypical patients, placing them within different categories. Steenkamp *et al*[46] refer to a group of patients who would meet some of the criteria described herein as diabetic (ketosis-prone diabetes), while other authors locate these patients within a subset of the LADA patients[47]. Overall, this reaffirms the necessity to continue the genetic analysis of this particular population to achieve a more adequate classification and treatment of these patients.

ARTICLE HIGHLIGHTS

Research background

In a high percentage of patients, clinical presentation alone does not define the type of diabetes. This is very important, since it hinders implementation of an individualized and safe treatment. The current classification system of diabetes is useful and easy for typical patients. However, there are many situations in which it is difficult to determine what type of diabetes is presenting due to the great heterogeneity in the pathogenesis. The current classification of diabetes is not satisfactory and its revision has been under consideration for many years. Previous studies carried out in the Uruguayan population have demonstrated the existence of patients for who it is not possible to classify into any of the categories provided in the international guidelines. We continue to investigate this type of patient because it is very important to assist them appropriately and improve their quality of life. In this way, it is possible to abolish the trial stage and error that patients suffer from when not being correctly diagnosed. At this time, different researchers have proposed that the classifications of diabetes should be revised, and this is the principal objective of our work. We have emphatically proposed the inclusion of genetics determination for HLA to elucidate atypical diabetes patients. Such an approach and related data will permit correct classification and treatment for these kinds of patients.

Research motivation

To date, we have investigated genes related to type 1 diabetes in patients with atypical diabetes. In this study, we sought to analyze the major gene related to

type 2 diabetes, the *TCF7L2* gene, in the atypical diabetes patients.

Research objectives

To analyze the association of the two most important single nucleotide polymorphisms (SNPs) of the *TCF7L2* gene-rs12255372 and rs7903146-with atypical diabetes.

Research methods

This case-control study was conducted in atypical and classical cases of type 2 diabetes using genotypification with *Taq*Man probes for the rs12255372 and rs7903146SNPs of the *TCF7L2* gene.

Research results

The SNPs of the *TCF7L2* gene that were analyzed in this work showed no association with atypical diabetes; nevertheless, the rs12255372 SNP was associated with classical diabetes.

Research conclusions

As has been shown in previous studies, the genetics of atypical diabetes are different from those of classical diabetes, despite a shared phenotype.

Research perspectives

To continue the characterization of the atypical diabetes subpopulation it will be important to obtain measurements of C-peptide in these patients and to study if there is any difference for this marker between the populations classified.

ACKNOWLEDGMENTS

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