

## Endothelial dysfunction as a predictor of cardiovascular disease in type 1 diabetes

Marcello C Bertoluci, Gislaïne V Cé, Antônio MV da Silva, Marco V Wainstein, Winston Boff, Marcia Puñales

Marcello C Bertoluci, Marco V Wainstein, Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul, Porto Alegre RS 90035-003, Brazil

Marcello C Bertoluci, Serviço de Medicina Interna do Hospital de Clínicas de Porto Alegre, Porto Alegre RS 90035-903, Brazil

Marcello C Bertoluci, Programa de Pós-Graduação em Ciências Médicas, Universidade Federal do Rio Grande do Sul, Porto Alegre RS 90035-003, Brazil

Gislaïne V Cé, Winston Boff, Marcia Puñales, Instituto da Criança com Diabetes - Grupo Hospitalar Conceição, Porto Alegre RS 91350-250, Brasil

Antônio MV da Silva, Departamento de Fisioterapia e Reabilitação, Universidade Federal de Santa Maria, Santa Maria RS 97105-900, Brasil

Marco V Wainstein, Serviço de Cardiologia do Hospital de Clínicas de Porto Alegre, Porto Alegre RS 90035-903, Brazil

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Correspondence to: Marcello C Bertoluci, MD, DMSc, Serviço de Medicina Interna do Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcellos 2350- Sala 700, Santa Cecília, Porto Alegre 90035-003, Brazil. [mbertoluci@uol.com.br](mailto:mbertoluci@uol.com.br)  
Telephone: +55-51-33598000  
Fax: +55-51-33598000

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### Abstract

Macro and microvascular disease are the main cause of morbi-mortality in type 1 diabetes (T1DM). Although there is a clear association between endothelial dysfunction and atherosclerosis in type 2 diabetes, a cause-effect relationship is less clear in T1DM. Although endothelial dysfunction (ED) precedes atherosclerosis, it is not clear whether, in recent onset T1DM, it may progress to clinical macrovascular disease. Moreover, endothelial dysfunction may either be reversed spontaneously or in response to intensive glycemic control, long-term exercise training and use of statins. Acute, long-term and post-prandial hyperglycemia as well as duration of diabetes and microalbuminuria are all conditions associated with ED in T1DM. The pathogenesis of endothelial dysfunction is closely related to oxidative-stress. NAD(P)H oxidase over activity induces excessive superoxide production inside the mitochondrial oxidative chain of endothelial cells, thus reducing nitric oxide bioavailability and resulting in peroxynitrite formation, a potent oxidant agent. Moreover, oxidative stress also uncouples endothelial nitric oxide synthase, which becomes dysfunctional, inducing formation of superoxide. Other important mechanisms are the activation of both the polyol and protein kinase C pathways as well as the presence of advanced glycation end-products. Future studies are needed to evaluate the potential clinical applicability of endothelial dysfunction as a marker for early vascular complications in T1DM.

**Key words:** Endothelial dysfunction; Type 1 diabetes; Cardiovascular disease

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**Core tip:** Endothelial dysfunction is an early finding in the natural history of type 1 diabetes and is predictive for microvascular disease and premature atherosclerosis. Decreased nitric oxide due oxidative stress is the central pathogenetic mechanism. Polyol pathway activation, protein kinase C (PKC) activation and advanced glycation product formation are also important. Long-term hyperglycemia, repeated hypoglycemia and microalbuminuria are factors associated. Intensive glycemic control and exercise training ameliorate endothelial dysfunction. Statins and renin-angiotensin system blockers are partially effective and may be influenced by hyperglycemia. There is a possible clinical benefit for the use of vitamin E and vitamin C that are still to be confirmed. PKC inhibitors are still investigative.

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## INTRODUCTION

Micro and macrovascular complications are leading causes of morbidity and mortality in patients with type 1 diabetes mellitus (T1DM)<sup>[1,2]</sup>. Subjects with T1DM are prone to accelerated atherosclerosis<sup>[3]</sup> and have 3 to 6 times more risk of cardiovascular death than individuals without diabetes. Endothelial dysfunction (ED) is an early event along the natural history of T1DM, indicating a phenotype in risk for accelerated atherosclerosis, that may be independent of the classical cardiovascular risk factors<sup>[4,5]</sup>. Interestingly, traditional cardiovascular risk factors altogether can not explain the totality of the cardiovascular risk in T1DM<sup>[6,7]</sup>. Chronic hyperglycemia *per se*, although an important predictor of microvascular disease, is a weak predictor of macrovascular complications in both T1DM and T2DM<sup>[8,9]</sup>. Thus, much of the residual cardiovascular risk still remains unexplained. In this context, ED becomes a new important risk factor that should be debated in the cardiovascular scenario. In the present review, we discuss recent evidences in the pathogenesis of endothelial dysfunction, its role as a risk factor for cardiovascular disease and the potential interventions for reducing endothelial dysfunction in T1DM.

## THE NORMAL ENDOTHELIUM

The vascular endothelium forms the cell layer that is

directly in contact with the vascular lumen, separated from the smooth muscle layer of the basement membrane. Its role is to maintain the homeostasis between the blood and the arterial wall through the synthesis of substances that modulate vascular tone, inhibit platelet aggregation and control the proliferation of vascular smooth muscle cells<sup>[10]</sup>.

In endothelial cells, nitric oxide (NO) is essential for the maintenance of integrity and homeostasis of endothelium<sup>[11]</sup>. NO is synthesized from L-arginine by the action of endothelial nitric oxide synthase (eNOS) in the presence of oxygen, NADP(H) and the NOS co-factor, tetrahydrobiopterin (BH4)<sup>[12]</sup>. The synthesized NO diffuses itself quickly into the smooth muscle cell layer and into platelets where it activates guanylate cyclase (GCA), with consequent production of cyclic GMP (cGMP). The presence of cGMP, in turn, promotes vascular relaxation and inhibition of platelet aggregation, keeping the equilibrium between pro and anti-thrombotic factors in the blood and arterial wall. However, as the half-life of NO is very brief, rapidly oxidizing into nitrate, the continuous activation of eNOS becomes the key determinant of NO synthesis and tissue bioavailability<sup>[11]</sup>. Normally, eNOS is activated by the turbulent blood flow against the luminal endothelial wall (shear-stress) as well as by the stretch of vascular wall cells and changes in the oxygen tension, promoting vascular muscle relaxation, an effect known as "endothelial-dependent vasodilation"<sup>[13,14]</sup>.

The stability of the endothelium is also dependent on endothelial repair and regeneration, which are determined by migration and proliferation of surrounding mature endothelial cell resident the vascular wall<sup>[15]</sup>. Recently it has been demonstrated that circulating endothelial progenitor cells (EPCs) are important in the endothelial regeneration. EPCs are circulating bone-marrow-derived cells characterized by the expression of varying surface markers that adhere to the damaged endothelium promoting tissue repair<sup>[15]</sup>. Circulating EPCs are considered biomarkers of endothelial function and prognostic indicators of cardiovascular morbi-mortality. Endothelial dysfunction represents the breakdown of this endothelium homeostasis, leading to a pro-thrombotic and pro-inflammatory that may lead to progressive atherosclerosis.

## METHODS FOR ASSESSMENT OF ENDOTHELIAL FUNCTION

Endothelial function can be investigated through invasively and non-invasively techniques. Coronary arteries can be evaluated invasively through angiography with quantitative measurement of changes in the vascular diameter in response to infusion of acetylcholine<sup>[16]</sup> and also through invasive venous occlusion plethysmography<sup>[17]</sup>, which measures forearm blood flow in response to acetylcholine infusion in the brachial artery. The invasive nature of

these techniques, involving artery cannulation and infusion of vasoactive drugs<sup>[14]</sup>, make them unfeasible to widespread use in clinical practice.

Non-invasive techniques, on the other hand, are being increasingly used in clinical settings. The flow mediated dilation (FMD) is the most popular technique currently used<sup>[18-20]</sup>. The rationale in FMD is based on reactive hyperemia responsive to shear-stress caused by turbulent blood flow, causing NO to be released and promoting endothelium-dependent vasodilation<sup>[14,21]</sup>. The measurement of vascular dilation can be done by capturing images of the brachial artery using high-resolution ultrasound<sup>[18]</sup>. Reactive hyperemia occurs after a period of ischemia, induced by occlusion of the brachial artery, with a sphygmomanometer cuff inflated with progressive release of vasodilator mediators such as adenosine and <sup>+</sup>H ions from ischemic tissue. When the release of blood flow occurs, sudden shear-stress is produced in the brachial vein endothelium, which is a strong stimulus for releasing NO<sup>[19]</sup>. This mechanism depends on the integrity of eNOs. The lesser the dilation the more severe the dysfunction. Definition of ED is than considered arbitrarily when the increase in dilation is less than 8%<sup>[20]</sup>.

Other non-invasive techniques are used less frequently. Peripheral artery tonometry induced by reactive hyperemia<sup>[22]</sup> assesses endothelial function by a combination of flow-mediated dilation and measurement of the amplitude of the arterial pulse wave expansion through a pneumatic sensor placed on the index finger. The microvascular reactivity on the forearm skin is evaluated with laser Doppler flowmetry, being the the iontophoresis of acetylcholine the endothelium-dependent vasodilator stimulus. The determination of the complacency of the dorsal hand vein is a minimally invasive method described by Aellig<sup>[23]</sup> in 1981 which has been used by our group<sup>[24]</sup>. It consists in a infusion of vasoactive drugs into the vein surface of the dorsum of the hand to measure endothelium-dependent vasodilation in response to acetylcholine, bradykinin or isoproterenol. The venous occlusion plethysmography can also be used to measure changes in forearm blood flow in response to reactive hyperemia. Finally, the measurement of the thickness of the intima-media layer (IMT) of the common carotid by ultrasound is a structural marker of atherosclerosis and correlates inversely with FMD in the brachial artery<sup>[25]</sup>. Increases in the IMT are indicative of early atherosclerosis.

## ENDOTHELIAL CHANGES IN DIABETES

Chronic sustained hyperglycemia in diabetes promotes important structural and functional modifications in the endothelium, as reported in both experimental and clinical studies<sup>[26-29]</sup>. In the aorta of rabbits with alloxan-induced diabetes, endothelial changes are visible after 2 wk from the onset of hyperglycemia

and become more severe after 6 wk of the diabetes onset<sup>[26]</sup>. The findings include adhesion of leukocytes, platelets and fibrin material on the endothelial surface. In mice, 6 wk after the onset of diabetes induced by streptozotocin (STZ), it is possible to observe increased endothelial permeability and endothelial cell apoptosis<sup>[27]</sup>.

In a classic study using samples of human skin and subcutaneous tissue obtained from autopsies and biopsies of 24 patients with T1DM, which were compared to 9 non-diabetic controls, the most important finding was the increase in the thickness of the basement membrane in T1DM patients compared with non-diabetic subjects<sup>[28]</sup>. In another study<sup>[29]</sup>, the increased thickness of the capillary basement membrane of skeletal muscle from patients with 12 years of T1DM, could be reversed by intensive glycemic control during one year<sup>[29]</sup>. In studies using electron microscopy, endothelial cells obtained from umbilical cord blood from pregnant women with T1DM show increased mitochondrial area when compared to pregnant women without diabetes<sup>[28]</sup>. The clinical significance of these structural changes, however, is still not clear to predict future atherosclerosis.

Important functional changes occur in the endothelium of T1DM. Hyperglycemia induces excess of electrons that leak from the oxidative chain and are captured by oxygen, generating superoxide excess and oxidative stress. Excessive superoxide production uncouples eNOS, impairing NO production<sup>[29]</sup>. The net effect is a reduction of NO production in response to shear stress in the inner vascular wall. By this way, the main determinant of ED is the preponderance of vasoconstrictor factors released by the endothelium in detriment of vasodilators factors due to the decreased availability of NO<sup>[30]</sup>. The dysfunctional endothelium leads to a migration of blood cells into the arterial wall, inducing proliferation of smooth muscle cells, platelet aggregation, LDLc oxidation, monocyte adhesion and synthesis of inflammatory cytokines, all factors contributing to atherogenesis<sup>[31]</sup>.

In patients with T1DM, functional changes in endothelium occurs very early in the natural history of diabetes<sup>[32]</sup>. The duration of diabetes is a major determinant for the presence of endothelial dysfunction in T1DM, being inversely correlated with the endothelium-dependent dilation. ED generally occurs in the first decade of T1DM, earlier than increases in the carotid intima-media layer thickness (Table 1).

ED is a common finding in T1DM, generally seen after 4 years of disease. In the study by Singh *et al*<sup>[33]</sup>, 31 adolescents with 6.8 years of T1DM and poor glycemic control presented both ED and increased intima-media layer thickness of carotid artery, compared with individuals without diabetes. The duration of diabetes was inversely correlated with the endothelium-dependent dilation<sup>[33]</sup>. These results were confirmed by other authors<sup>[34-38]</sup> and are in accordance

**Table 1 Studies on Flow-Mediated Dilatation and Nitroglycerin Mediated Dilatation in patients with type 1 diabetes**

Ref.	n	Age	Time	Micro-albuminuria	HbA1c	FMD		NTG	
						Type 1 diabetes	Non-diabetes	Type 1 diabetes	Non-diabetes
Clarkson <i>et al</i> <sup>[36]</sup>	80	15-40	13	6%	9.5	5.8 ± 3.7 <sup>a</sup>	9.3 ± 3.8	15.6 ± 5.6 <sup>a</sup>	19.7 ± 6.6
Lambert <i>et al</i> <sup>[37]</sup>	52	32	15	No	7.9	12.9 ± 9.8	17.3 ± 9.9	14.3 ± 8.0	17.7 ± 8.7
Enderle <i>et al</i> <sup>[38]</sup>	17	41.5	21	No	8.0	8.2 ± 4.6	7.6 ± 4.2	16.3 ± 4.9	18.4 ± 6.4
Lekakis <i>et al</i> <sup>[35]</sup>	31	32	NO: 13 MA: 20	Yes	N: 6.5 M: 7.1	NO: 5.8 ± 7 <sup>a</sup> MA: 0.7 ± 2.5 <sup>a</sup>	11.0 ± 7 -	NO: 19.0 ± 6.9 <sup>a</sup> MA: 15.0 ± 2.9 <sup>a</sup>	24.0 ± 9.0
Dogra <i>et al</i> <sup>[34]</sup>	34	NO: 44 MA: 48	NO: 20 MA: 25	Yes	8.5-8.7	NO: 5.4 ± 0.6 <sup>a</sup> MA: 3.2 ± 0.3 <sup>a</sup>	7.9 ± 0.6 -	MA: 11.9 ± 1.1 <sup>a</sup>	20.0 ± 1.2
Singh <i>et al</i> <sup>[33]</sup>	31	15	7	No	8.6	4.2 ± 3.8 <sup>a</sup>	8.2 ± 4.2	17.0 ± 6.0	18.0 ± 6.0
Järvisalo <i>et al</i> <sup>[4]</sup>	45	11	4	No	8.9	4.4 ± 3.4 <sup>a</sup>	8.7 ± 3.3	-	-
Ladeia <i>et al</i> <sup>[39]</sup>	18	13	3	80%	9.3	10.9 ± 2.0	11.2 ± 2.4	-	-
Cé <i>et al</i> <sup>[5]</sup>	57	17	7	No	8.6	9.5 ± 6.5 <sup>a</sup>	14.6 ± 5.6	22.3 ± 9.8 <sup>a</sup>	29.3 ± 4.2

Age: Mean age in years; Time: Time of T1DM (in years); HbA1c: Hemoglobin A1c (%); NO: Normoalbuminuria; MA: Microalbuminuria; FMD: Flow-Mediated Dilatation; NTG: Nitroglycerin Mediated Dilatation. <sup>a</sup>*P* < 0.05 *vs* controls.

to the concept that endothelial dysfunction is predictive of early atherosclerosis in T1DM.

More recent data indicate that ED can occur even before 4 years of onset of T1DM<sup>[4,39]</sup>, preceding the onset of microalbuminuria. Järvisalo *et al*<sup>[4]</sup> compared non-obese, poor-controlled, recent onset T1DM children with age-matched children without diabetes, with respect to FMD and the thickness of intima-media carotid. They observed the presence of endothelial dysfunction in 36% of cases, a lower peak of flow mediated dilation response and increased intima-media thickness compared with controls. The authors concluded that ED is a common finding in children in the early years of T1DM and may be a predictor for the development of premature atherosclerosis.

The presence of ED, however, is not uncommon before 4 years of T1DM<sup>[32]</sup>. We found a prevalence of 35.7% of ED in a sub-group of T1DM patients with less than 5 years of diabetes<sup>[5]</sup>. The data from the above studies indicates that it ED may begin to occur 3 to 5 years from the onset of T1DM.

## FACTORS ASSOCIATED WITH ED IN T1DM

### Gender

The impact of gender in ED is still undefined, but, in one study, boys with T1DM seemed to be at increased risk. Bruzzi *et al*<sup>[40]</sup> studied 39 children with T1DM and 45 healthy age-matched controls, evaluated longitudinally with FMD at baseline and 1 year of follow-up<sup>[40]</sup>. At baseline, T1DM boys and girls had similar FMD values, however, after 1 year, boys had more endothelial dysfunction than girls. The rationale of this difference is still unknown since multivariate analysis did not identify important predictors of endothelial dysfunction<sup>[40]</sup>.

### Acute hyperglycemia

Acute hyperglycemia is capable to induce reversible

endothelial dysfunction in normal individuals. When non-diabetic subjects are acutely exposed to high concentrations of glucose during dextrose infusion for 6 h, there is an attenuation of the arterial endothelium-dependent vasodilation induced by methacholine (endothelium-dependent vasodilation) while preserving the vasodilator response to nitroprusside (non-endothelium dependent vasodilation)<sup>[41]</sup>. This indicates that acute rises in blood glucose in contact to a previous normal endothelium can cause acute endothelial dysfunction, but it is not sufficient to promote vascular smooth muscle dysfunction. In another study in normal subjects<sup>[42]</sup>, it was also demonstrated that acute hyperglycemia can cause significant hemodynamic and rheological changes such as increases in systolic and diastolic blood pressure, heart rate and plasma catecholamines, while decreasing arterial blood flow to the leg. Platelet aggregation to ADP and blood viscosity also showed increments. When the authors infused the natural precursor of NO formation, L-arginine, blood pressure and artery flow changes were reversed. When they infused the inhibitor of endogenous NO synthesis, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), hemodynamic effects of hyperglycemia were reproduced, indicating that acute hyperglycemia reduces NO availability even in normal subjects<sup>[42]</sup>.

The effect of acute high glucose in normal endothelium, however, is not observed in all studies. Houben *et al*<sup>[43]</sup> studied the effect of acute glucose infusion for 24 h in normal individuals and did not observed changes in vascular dilatation of skin microcirculation induced by acetylcholine (Ach), nitroprusside, norepinephrine or nitric oxide synthase antagonist (L-NMA). The differences between studies may be due to methodological differences. It is accepted, however, that insulin can attenuate acute endothelial dysfunction, promoting compensatory vasodilation which may have biased the studies. In other studies, where the action of insulin was blocked

by octreotide, the effect of acute hyperglycemia alone was evident<sup>[41]</sup>.

### **Long-term hyperglycemia**

The association between HbA1c and flow-mediated dilation (FMD) is seen in some cross-sectional studies with patients with T1DM. In the study of Ladeia *et al.*<sup>[39]</sup>, with 19 normo and microalbuminuric T1DM patients, there was a moderate positive correlation between FMD and HbA1c. In another study<sup>[44]</sup>, patients with T1DM with HbA1c above 6.0% had significant impairment of endothelial function compared to patients with HbA1c below 6%, indicating that chronic mild increases in mean hyperglycemia are also associated to ED in T1DM.

We studied the impact of chronic glycemic control in endothelial function of T1DM in a historical cohort study<sup>[5]</sup>. T1DM adolescents under 5 year of disease were evaluated for ED and had their mean HbA1c obtained from medical records in the same institution since their diagnosis. Considering as a whole, the mean historical HbA1c was clearly higher in T1DM patients with endothelial dysfunction compared with T1DM without ED. Interestingly, we observed a moderate inverse correlation between FMD and the historical mean of HbA1c in the first 2 years after the diagnosis of T1DM but not with the more recent HbA1c. The plausible explanation was that endothelial function could be more affected by the long-term than by the short-term glycemic control, supporting the concept of metabolic memory<sup>[45]</sup>. Glycation of the endothelium in the first years of T1DM seems, by this way, decisive to determine future endothelial dysfunction in T1DM<sup>[32]</sup>.

### **Post-prandial hyperglycemia**

The effect of postprandial hyperglycemia in endothelial function was studied by Giugliano *et al.*<sup>[42]</sup> in individuals with type 2 diabetes. The combined effect of postprandial glucose and hypertriglyceridemia was associated with increased serum concentrations of adhesion molecules such as ICAM-1, E-selectin and VCAM-1. Markers of oxidative stress such as nitrotyrosine increased sharply after ingestion of 75 g of oral glucose. This effect was greater when an additional lipid overload was used, indicating that both acute hyperglycemia and hyperlipemia can affect endothelial function in diabetes<sup>[46]</sup>.

In another study, the same authors<sup>[47]</sup> observed that, after a glucose overload, ED accentuates until the second hour, but returns to basal level after 4 h from the beginning of the overload. Though, lipid overload did not change FMD until the fourth hour. These data suggests that the effect of postprandial glycaemia in endothelial function is independent of the postprandial lipemia, although both might be mediated by increased oxidative stress.

### **Hypoglycemia**

Recently, it was demonstrated that repeated episodes of hypoglycemia in subjects with T1DM may be associated with endothelial dysfunction and could be an aggravating factor for preclinical atherosclerosis. In a case-control study<sup>[48]</sup>, T1DM with repeated hypoglycemia episodes were compared with age and sex-matched T1DM controls who did not have frequent hypoglycemias. Vascular function was assessed by FMD, intimal-media carotid artery thickness (IMT) and endothelial dysfunction markers such as von Willebrand factor (vWf). The group with increased hypoglycemia episodes presented lower percentages of FMD in response to ischemia, increased IMT measures and higher endothelial function markers. In another study<sup>[49]</sup>, with cross-sectional design, T1DM children and age and gender-matched healthy children were evaluated for vascular function and continuous glucose monitoring system (CGMS) in order to compare the impact of glucose variability and hypoglycemia episodes in vascular function. Subjects with T1DM had significantly lower FMD compared to healthy children. However, when comparing CGMS parameters, the authors found significant inverse relationship between FMD with hypoglycemia indexes but not with variability indexes.

The biological rationale for hypoglycemia inducing endothelial dysfunction in T1DM is that acute hypoglycemia can induce rapid pro-inflammatory, platelet aggregatory, anti-fibrinolytic response, and recurrent hypoglycemia may induce changes in hemostatic factors and viscosity which may decrease perfusion in diabetic microangiopathy<sup>[50]</sup>.

### **Glycemic variability**

Glycemic variability (GV) is a term exclusively related to blood glucose fluctuations and must be differentiated from post-prandial glucose (PPG). GV is related to glucose variability along the day, while PPG is related to the precise time of glucose rise after a meal. PPG effect in vascular function can also be influenced by other confounders such as hypertriglyceridemia.

Whether GV is an important factor to cause ED is still a matter of debate. The effect of glycemic variability in ED was studied in T1DM with normal urinary albumin excretion<sup>[51]</sup>. Patients were exposed to 48 h of good (mean 113 mg/dL) or poor metabolic control (mean 286 mg/dL) and evaluated with FMD and serological markers of endothelial function. Both endothelium-dependent and endothelium-independent vasodilation were significantly impaired after the poor control period in relation to good glycemic control. There was also a significant increase in vWf levels after the deterioration of control. These results indicate that endothelial function may suffer significant impact of acute variability of blood glucose in T1DM, although it

can be reversed with improvement of glycemic control. However this effect was not seen in other studies. In the DCCT study, the GV in glycemic data using 7-point self monitoring blood glucose (SMBG) obtained every 3 mo did not correlated to macrovascular complications<sup>[52]</sup>. We were also not able to detect a relationship between FMD and the standard deviation (SD) of glycemia in T1DM, using day 7-point SMBG for 30 d preceding FMD (data unpublished). Finally, in a cohort study of T1DM<sup>[53]</sup>, the standard deviation of blood glucose, calculated from self-monitoring blood glucose data along 11 years of follow up, was predictive for incident peripheral neuropathy<sup>[53]</sup>. The influence of GV in vascular function and future micro or macro vascular complications of T1DM is not yet established.

### Microalbuminuria

Microalbuminuria is strongly associated with ED in T1DM. Dogra *et al.*<sup>[34]</sup> studied long-term T1DM patients with microalbuminuria with poor glycemic control who were compared to normoalbuminuric T1DM and to non-diabetic individuals. FMD was more severely impaired in T1DM patients with microalbuminuria being albuminuria an independent predictor of ED. In another study, Lekakis *et al.*<sup>[35]</sup> observed lower FMD values in microalbuminuric compared with normoalbuminuric patients. In a similar study with children and adolescents with T1DM with less than 5 years of disease<sup>[39]</sup>, there was a negative correlation between the percent of endothelium-mediated dilation and albuminuria. Mean FMD was also significant decreased in microalbuminuric. ED is also be present in type 2 diabetes patients with normal albuminuria with long duration T2DM during chronic poor glycemic control<sup>[54]</sup>. In T1DM patients with normoalbuminuria ED is less common<sup>[40]</sup>.

## MECHANISMS OF ED IN T1DM

### Oxidative stress

Children with T1DM have increased oxidative stress and reduction of anti-oxidant defense compared to healthy children and to their parents<sup>[55,56]</sup> and these results are similar in adolescents with T1DM<sup>[57]</sup>. Moreover, endothelial progenitor cells are also reduced in children with T1DM compared to non-diabetic controls possibly related to oxidative stress<sup>[58]</sup>.

Hyperglycemia can cause excessive production of superoxide in the mitochondria oxidative chain of endothelial cells. The excess of superoxide reacts rapidly with NO, reducing NO bioactivity and producing peroxynitrite (ONOO<sup>-</sup>). Peroxynitrite is a potent oxidant agent and an activator of the lipid peroxidation which may impair endothelial function by stimulating arachidonic acid metabolism<sup>[59]</sup>. The overproduction of superoxide and NO favors the formation of peroxynitrite by interfering with the production of the eNOS cofactor,

tetrahydrobiopterin (BH4)<sup>[60]</sup>.

NAD(P)H oxidase is a chief determinant enzyme of superoxide production in animal models of vascular diseases, including diabetes<sup>[32]</sup>. In arteries of patients with diabetes who were submitted to artery bypass surgery, it was demonstrated that the endothelium can produce superoxide induced by a dysfunctional eNOS. Dysfunctional eNOS is caused by the oxidation of the co-factor BH4 into BH2. The enzymatic uncoupling of eNOS in human endothelium turns eNOS into dysfunctional eNOS which promotes a transition from NO production to superoxide production<sup>[60,61]</sup>.

### Protein kinase C pathway activation

Protein kinase C (PKC) is a cytoplasmic family of enzymes with a wide variety of actions in intracellular signal transduction. The activation of PKC by decreases endothelium derived nitric oxide synthesis, whereas its inhibition increases NO release. The beta isoforms are activated in response to hyperglycemia<sup>[62]</sup>. The activation of PKC system is also associated with increased albuminuria in rats<sup>[63]</sup>.

There are several mechanisms in which PKC may decrease the bioavailability of NO. PKC antagonizes activation of eNOS, decreases NO concentration, and induces NAD(P)H oxidase to produce superoxide, which, in turn, uncouples eNOS, inducing the production of even more superoxide. PKC is associated with various vascular disorders such as a decrease of Na<sup>+</sup>/K<sup>+</sup> ATPase, increased extracellular matrix, increased vascular permeability, contractility and cell proliferation. The activation of PKC system is also associated with increased albuminuria in rats<sup>[63]</sup>.

In a randomized double-blind placebo controlled clinical trial, in healthy individuals submitted to acute hyperglycemia with hyperglycemic clamp technique, FMD was attenuated by hyperglycemia and reversed after treatment for 7 d with the PKC beta inhibitor, LY333531, indicating that the PKC-B system is an important regulator of hyperglycemia-induced endothelial dysfunction<sup>[64]</sup>.

### Advanced glycation products

In the presence of sustained hyperglycemia, tissue proteins such as collagen undergo non-enzymatic glycation and formation of cross-links, resulting in advanced glycation end-products (AGEs). AGEs promotes a permanent chemical modification of proteins, stimulating cellular responses through specific anti-proliferative receptors<sup>[65,66]</sup>. These receptors were first observed in experiments in mouse peritoneal macrophages<sup>[66]</sup>, showing ability to remove modified glycated proteins. AGEs may reduce the availability of endothelial NO, and reactive AGE intermediates may compromise their anti-proliferative effect.

**Polyol pathway activation**

Chronic hyperglycemia increases the activity of aldose reductase enzyme and leads to activation of polyol pathway, transforming glucose into sorbitol and subsequently into fructose. It also induces the consumption of NADP(H), an important cofactor for NO synthesis<sup>[61]</sup>. As NADP(H) is an important cofactor for NOS to NO synthesis, its depletion leads to reduction of NO production. It remains uncertain, however, the magnitude of importance in the prevention of human atherosclerosis.

**ED AS A MARKER OF CARDIOVASCULAR RISK IN T1DM**

Although endothelial dysfunction and chronic low-grade inflammation have been associated with atherothrombotic cardiovascular disease, independently of traditional cardiovascular risk factors in either individuals with or without diabetes, a clear cause-effect relationship with atherosclerosis is not yet established in the natural history of T1DM.

In non-diabetic patients with coronary disease, endothelial dysfunction is predictive for increasing risk of cardiovascular events<sup>[67]</sup>. In an observational study, 157 patients with mild coronary disease were classified according to the severity of ED, which was defined by intracoronary ultrasound with vascular reactivity after administration of acetylcholine, adenosine or nitroglycerin<sup>[67]</sup>. They were followed by a mean of 28 mo for the assessment of cardiovascular outcomes. At the end of follow up, patients with more severe ED presented 14% of cardiovascular events, while those with mild or no ED had no cardiovascular outcomes ( $P < 0.05$ )<sup>[67]</sup>. This study demonstrated, for the first time, that patients with mild coronary disease but with severe ED were at increased risk for cardiovascular events.

**Serum markers of ED**

The vWf and C-Reactive protein (CRP) are related to ED and inflammation. In the population-based cohort study, the HOORN study<sup>[68]</sup>, the predictive value of the serum ED marker, vWf, was evaluated for cardiovascular mortality in T2DM patients. The cohort including 2,484 caucasian individuals with ages between 50-70 years, in which 27% had T2DM and 27% had impaired glucose tolerance, was followed by 5 years<sup>[68]</sup>. Patients with vWf levels in the upper tertile had a 3 fold increase in cardiovascular mortality compared to those in the lower tertiles, even after adjustments for age, sex and glucose tolerance status. The relative risk for all-cause mortality associated with vWf was 2.03 (95%CI: 1.19 to 3.47). The predictive value of vWf was not confirmed in ARIC study<sup>[69]</sup>, however, vWf is also an independent predictor of cardiovascular mortality in specific populations<sup>[70]</sup>.

CRP is an inflammatory marker and can be increased in T1DM patients without clinical macroangiopathy, compared with healthy subjects<sup>[71]</sup>. This increase is greater in the presence of micro or macroalbuminuria compared with normoalbuminuric patients indicating an association between endothelial dysfunction and vascular inflammation<sup>[71]</sup>.

The mechanisms by which the cardiovascular risk is associated with elevated levels of vWf and CRP are not completely understood. It may reflect generalized endothelial dysfunction, increased prothrombotic state<sup>[71]</sup>, inflammation and greater risk for developing atherosclerosis<sup>[72]</sup>. Von Willebrand factor in combination with *t*-PA measurement may also be an index for endothelial dysfunction<sup>[73]</sup>.

Markers of endothelial function can also be determinants of inflammation. In the EURODIAB Prospective Complications Study<sup>[74]</sup>, a nested case-control study of 543 T1DM participants, the levels of serum markers of ED such as E-selectin, vascular adhesion molecule-1 cell (VCAM-1) and inflammatory markers were determined. In this study, endothelial dysfunction was strongly associated with inflammatory activity suggesting that endothelial dysfunction may interact with vascular inflammation in T1DM which potential consequences in accelerating atherosclerosis<sup>[74]</sup>.

**Flow mediated dilation**

Impaired flow mediated dilation (FMD) may also predispose to early atherosclerosis in T1DM patients, as seen by the development of increased carotid artery thickness (IMT). In a cross-sectional study, 45 children with T1DM and 30 healthy matched in age, gender and body size were evaluated for FMD and IMT<sup>[75]</sup>. Children with diabetes presented lower peak FMD response and increased IMT compared to non-diabetic children. In another cross-sectional study<sup>[75]</sup>, T1DM adolescents without diabetes complications were compared with healthy age-matched controls in respect to FMD and the presence of diastolic dysfunction with pulse wave Doppler and tissue Doppler echocardiography measurements. ED was associated with segmental diastolic dysfunction. These studies suggest that ED is associated with indirect evidences of premature atherosclerosis, however, long-term prospective studies are still needed to conclude if FMD is predictive for cardiovascular events in early T1DM.

**CLINICAL MANAGEMENT OF ED IN T1DM****Intensive insulin therapy and glycemic control**

There are compelling evidences indicating that optimizing glycemic control with intensive insulin therapy reduces the development and progression of microvascular complications<sup>[1]</sup>. Although endothelial

dysfunction and oxidative-stress are early changes in T1DM, both conditions are only partially reversed by insulin therapy alone<sup>[76]</sup>. In adults with 8 years of T1DM who are in poor glycemic control, acute intravenous insulin infusion can only partially reverse impaired FMD, even after completely normalizing glycaemia. It is likely that a more prolonged treatment is necessary for achieving a reversion to normal functioning of endothelium-dependent vasodilation in T1DM<sup>[76]</sup>.

In a clinical trial, 92 children and adolescents with T1DM in conventional insulin therapy were randomized for either continuing in conventional insulin therapy or to switch to a more intensive insulin therapy, including insulin infusion pump and multiple insulin injections. After 1 year of intensive insulin therapy, the baseline vascular response to acetylcholine and the levels of E-selectin improved significantly in the intensive group, while no effect was seen in the conventional group. Interestingly, in this study the benefit was independent of HbA1c, suggesting that intensive insulin therapy may confer vascular protection in addition to improving glycemic control<sup>[76]</sup>.

### Exercise

Exercise has a great impact in the mitigation of ED in patients with cardiovascular risk factors both in T1DM and T2DM. In children with T1DM, 30 min of aerobic training, two times a week, for 18 wk can significantly increase flow mediated dilation in around 65%<sup>[77]</sup>. In adults with T1DM, 60 min of aerobic training, 2 times a week, significantly improves flow mediated dilation in more than 50% after 24 wk<sup>[78]</sup>. In a cross sectional study, T1DM adolescents who did more than 60 min daily of moderate-to-vigorous physical activity have higher flow mediated dilation than inactive patients with diabetes<sup>[79]</sup>. Many other studies also show improvement of vasodilator response in T2DM without coronary artery disease, with both aerobic and mixed aerobic/resistance training with 8 to 12 wk of duration<sup>[80-82]</sup>.

The main mechanism underlying the amelioration of vasodilation in response to exercise is largely related to the increased in nitric oxide (NO) bioavailability, resulted from the increased activity and expression of the eNOS and the diminished degradation of NO due to the action of radical oxygen species (ROS). Cultured cells experiments<sup>[83]</sup> indicate that shear-stress can induce eNOS expression and activity due to stabilization eNOS mRNA or increasing its synthesis. In humans with coronary artery disease, it was also demonstrated a two-fold increase in eNOS expression and a 3.2 increase in the phosphorylation of eNOS after 4 wk of regular training<sup>[84]</sup>. The increase in antioxidant defenses such as the activity of superoxide dismutase and glutathione peroxidase is another important mechanism underlying the improvement of endothelial function by exercise seen in patients with

heart failure<sup>[85]</sup>.

Exercise training can increase the number of circulations EPCs in healthy subjects<sup>[86]</sup> and coronary artery disease patients<sup>[87]</sup> after 4 wk of regular training. EPCs are decreased in patients with T1DM compared to healthy subjects<sup>[83,88]</sup>. The effect of exercise impact in EPCs in type 1 patients, however, remains to be clarified.

### Anti-oxidants

Experimental studies demonstrated that antioxidants can modulate the response of the endothelium dependent vasodilation, endothelium-leukocyte interactions and the balance of pro and anti-thrombotic factors<sup>[89-91]</sup>.

In clinical studies, treatment with oral high-dose vitamin E during 6 mo have yielded conflicting results in ED improvement in patients with T1DM. In the first study, Skyrme-Jones *et al.*<sup>[92]</sup> compared the effect of vitamin E 1000 UI/d with placebo in a double-blind randomized clinical trial in patients with T1DM in FMD. They found significant increases in FMD after 3 mo in the group receiving vitamin E. They considered that vitamin E decreased the LDLc oxidant capacity, thus reducing ED. In a double-blind randomized clinical trial in adults with both T1DM and T2DM, Beckman *et al.*<sup>[93]</sup> compared the use of a combination of vitamin E (800 UI/d) with vitamin C (1000 mg/d) compared to placebo. After 6 mo, FMD increased significantly in the T1DM group but not in T2DM. These data are promising, however, neither all studies confirm these findings. In a randomized clinical trial, Economides *et al.*<sup>[94]</sup> studied the effect of high-dose vitamin E (1800 UI) against placebo in T1DM and T2DM along 12 mo but failed to find improvements in ED<sup>[94]</sup>. The clinical effectiveness of vitamin E in improving ED and reducing the progression to atherosclerosis remains to be established in larger trials in T1DM.

Ascorbic acid infused together with intravenous insulin with near normalization of glycaemia, can rapidly normalize endothelial dysfunction in T1DM<sup>[75]</sup>. This effect is not completely attained, however, when either intensive glycemic control with insulin or ascorbic acid infusions are used alone, indicating that an additive effect of both treatments exist, an effect that may be limited to new-onset type 1 diabetes<sup>[89]</sup>. Ascorbic acid can also decrease transcapillary albumin escape<sup>[95]</sup> and urinary albumin excretion in T1DM adults<sup>[96]</sup>. In children with T1DM, the combined use of ascorbic acid and vitamin E can increase superoxide-dismutase levels<sup>[57]</sup>.

Low ingestion of antioxidants, especially vitamins, is associated with increased risk of cardiovascular disease and atherosclerosis<sup>[97,98]</sup>. The inverse correlation between concentrations of antioxidant agents, vitamins and disease risk could be associated to higher requirement of antioxidant molecules during inflammatory diseases.



Insufficient supply with these compounds may further accelerate disease process<sup>[99]</sup>. On the other hand, these data are not yet been convincingly established in clinical trials and are still controversial<sup>[100-102]</sup>.

### Statins

Statins may have benefic effect on endothelial dysfunction in patients with T1DM. In a clinical trial with 204 long-term T1DM randomized to receive atorvastatin 40 mg plus hypolipemic diet or placebo plus hypolipemic diet for 6 mo, FMD increased 44% and PAI-1 was reduced in atorvastatin group compared to placebo<sup>[103]</sup>. Similar results were observed in a small cross-over trial including 16 T1DM with microalbuminuria<sup>[104]</sup> who received atorvastatin 40 mg or placebo for 6 wk with a 4-wk period of washout. FMD and non-endothelium dependent vasodilation increased significantly while using atorvastatin. In a meta-analysis of 10 studies including 845 patients with both T1DM and T2DM<sup>[105]</sup>, statin therapy significantly ameliorates FMD in patients with diabetes, although heterogeneity among trials was found. Statins however, improved FMD only in patients with better endothelial function. Factors associated with improvements were: T1DM, younger age, lower baseline lipid levels and blood pressure. Mechanisms enrolled in this effect are not completely known but may be related to reductions in LDLc as well as pleiotropic anti-oxidant effects of statins.

### ACE inhibitors

Experimental evidences suggest that ACE inhibition may have benefic effects to the endothelium *in vitro*<sup>[106,107]</sup>. In a clinical trial, quinapril showed benefit in coronary endothelial function of non-diabetic patients with CAD<sup>[108]</sup>. ACE inhibitors improve FMD in T1DM with microalbuminuria although not in T1DM with normoalbuminuria<sup>[109]</sup>. In another small trial with normotensive T1DM patients with microalbuminuria, ACE-I inhibitors improved both FMD and GTN in the femoral artery after 1 wk of treatment<sup>[110]</sup>.

The direct renin blockade was studied in normoalbuminuric T1DM with the use of aliskiren during 4 wk of monotherapy, followed by 4 wk of a combination of aliskiren and ramipril. In both conditions of hyperglycemia and euglycemia of short duration, obtained through euglycemic clamp and hyperglycemic clamp techniques, ED ameliorated with aliskiren alone and improved further when in combination with ramipril. This effect only occurred in the euglycemic phase of the study. Effects were abolished when patients became hyperglycemic<sup>[111]</sup>.

### PKC inhibitors

Ruboxistaurin (RBX) is an orally administered isoform-selective inhibitor of PKC which was demonstrated to have beneficial effect in experimental models

of diabetic retinopathy<sup>[112]</sup> and in hemodynamic retinal abnormalities of patients with diabetes<sup>[113]</sup>. The studies PKC-DRS<sup>[114]</sup> and PKC DRS2<sup>[115]</sup> showed a 50% reduction in vision loss of patients treated with RBX. The effect of RBX was than studied in 2 combined phase 3 trials: MBDL and MBCU. Both were randomized, double-blind, placebo-controlled, clinical trials, including T1DM and T2DM with ages above 18 years. Patients had HbA1c below 11%, blood pressure below 160/90 mmHg and diabetic retinopathy. Patients were submitted to pan-photocoagulation or focal photocoagulation after randomization. Patients were than randomized to RBX 32 mg or placebo and followed by 36 mo (in MBDL) and 48 mo (in MBCU). Altogether, 1040 patients were randomized. Sustained moderate visual loss occurred in 4.4% of placebo vs 2.3% of RBX treated patients ( $P = 0.045$ ). The results were promising, indicating a potential reduction in visual loss of 50% above standard care<sup>[116]</sup>. RBX has also been studied in diabetic neuropathy in other smaller studies. In a 6 mo clinical trial, RBX vs placebo in patients with both T1DM and T2DM. Patients who were randomized for RBX presented improvement of neuropathic symptoms and ameliorated decreased skin microvascular blood flow<sup>[117]</sup>. Although promising, RBX is not available for clinical use.

## CONCLUSION

ED should be a concern for clinicians as an early and common phenomenon in T1DM, which may be predictive for future microvascular disease and early atherosclerosis. The clinical use of endothelial function measurement in clinical practice, specially FMD, is a potential tool to enhance cardiovascular risk prediction. Long-term intensive insulin treatment with optimized glycemic control along with exercise training are essential to prevent ED in these patients. Drugs such as statins and ACE-inhibitors are partially effective and may be influenced by the degree of hyperglycemia, with better response in microalbuminuric patients. There is also a possible benefit in using anti-oxidants such as vitamin E and vitamin C, but there is a clear demand for long-term randomized clinical trials to define their role in ED treatment. New agents such as PKC inhibitors are still investigative, but hold promise for future treatment of ED in T1DM.

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