Increased Plasma Levels of Endothelin 1 and von Willebrand Factor in Patients With Type 2 Diabetes and Dyslipidemia

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OBJECTIVE — Endothelial markers endothelin 1 (ET-1) and von Willebrand factor (vWF) were assessed in patients with type 2 diabetes and dyslipidemia and in patients with hypercholesterolemia.

RESEARCH DESIGN AND METHODS — In this case-control study, plasma ET-1 and vWF levels were measured by enzyme-linked immunosorbent assay in 35 normoalbuminuric type 2 diabetic patients with dyslipidemia (56 ± 5 years), in 21 nondiabetic patients with hypercholesterolemia (52 ± 7 years), and in 19 healthy control subjects (45 ± 4 years). All of the individuals were normotensive and nonsmokers. Urinary albumin was measured by immunoturbidimetry.

RESULTS — ET-1 levels were higher ($P < 0.0001$) in type 2 diabetic dyslipidemic patients (1.62 ± 0.73 pg/ml) than in both nondiabetic hypercholesterolemic patients (0.91 ± 0.73 pg/ml) and control subjects (0.69 ± 0.25 pg/ml). vWF levels were significantly increased ($P = 0.02$) in type 2 diabetic (185.49 ± 72.1%) and hypercholesterolemic (163.29 ± 50.7%) patients compared with control subjects (129.70 ± 35.2%). In the multiple linear regression analysis, ET-1 was significantly associated (adjusted $r^2 = 0.42$) with serum triglyceride levels ($P < 0.001$), age ($P < 0.01$), insulin sensitivity index ($P < 0.02$), and albuminuria levels ($P < 0.04$). vWF levels were associated (adjusted $r^2 = 0.22$) with albuminuria ($P < 0.001$), fibrinogen levels ($P < 0.02$), and BMI ($P < 0.03$).

CONCLUSIONS — Compared with hypercholesterolemic patients, type 2 diabetic patients with dyslipidemia have increased levels of ET-1 and vWF, which may indicate more pronounced endothelial injury. These findings appear to be related to components of the insulin resistance syndrome.

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Diabetes is associated with an increased risk of atherosclerosis, and coronary artery disease is a major cause of death in patients with diabetes. High total cholesterol is also a major risk factor for coronary artery disease. The Multiple Risk Factor Intervention Trial demonstrated that age-adjusted incidence of coronary artery disease was 4-fold greater in diabetic patients compared with nondiabetic subjects for any cholesterol level (1). Taken together, these observations suggest that atherogenesis might be differently expressed in diabetes, owing perhaps to specific diabetic features or to the clustering of common risk factors present in this disease state. In fact, we have recently shown that the features of advanced atherosclerotic lesions in type 2 diabetic patients submitted to limb amputation were different than those of nondiabetic patients, because increased accumulation of tumor necrosis factor-α expression was observed in the first group (2).

Endothelial cell injury is an important feature that appears early in the pathogenesis of atherosclerosis (3). Hypercholesterolemia impairs endothelium-mediated vasodilatation in response to acetylcholine (4). Endothelial dysfunction has also been shown to occur in type 2 diabetes. Multiple mechanisms are likely involved; in fact, components of the insulin resistance syndrome may be central to the development of diabetic dysfunctional endothelium (5).

Different approaches (i.e., morphological, functional, and biologic) may be used to assess endothelial integrity. The plasma levels of biologic markers, such as the von Willebrand factor (vWF), endothelin 1 (ET-1), and adhesion molecules in different proatherogenic conditions (6,7), may reflect endothelial function. Increased levels of vWF, a glycoprotein synthesized by endothelial cells, in subjects with type 2 diabetes are associated with macrovascular mortality (8). ET-1, a vasoconstrictor and mitogenic endothelium-derived peptide, was found to be increased in patients with diabetes compared with healthy subjects, especially in patients with retinopathy, albuminuria, or macrovascular disease (9).

Even though both type 2 diabetes and lipid disorders are each clearly related to endothelial dysfunction, it is unclear whether the association of these conditions configures a higher threat to the endothelium. Specifically, the profile of ET-1 and vWF expression, as well as their possible association with different metabolic abnormalities present in diabetes and lipid disorders, remains poorly explained. Therefore, the aim of this study was to assess endothelial function by measuring ET-1 and vWF plasma levels in normotensive nonsmoking normoalbuminuric patients with type 2 diabetes and dyslipidemia, in hypercholes-

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Abbreviations: CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin 1; HOMA, homeostasis model assessment; UAE, urinary albumin excretion; vWF, von Willebrand factor; WHO, World Health Organization.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Clinical and laboratory data of subjects*

	Type 2 diabetic subjects with dyslipidemia	Hypercholesterolemic subjects	Control subjects	*P*
n	35	21	19	—
Age (years)	56 ± 5*†	52 ± 7*	45 ± 4	0.0001
Sex (F/M)	17/18	5/16	6/13	0.15
BMI (kg/m^2)	28.4 ± 3.6*	27.2 ± 3.2*	24.2 ± 2.2	0.0001
sBP (mmHg)	130.7 ± 11.2*†	121.8 ± 11.2	122.5 ± 9.3	0.002
dBP (mmHg)	76.9 ± 7.5	74.9 ± 6.8	72.5 ± 7.1	0.24
Glucose (mg/dl)	152 ± 21*†	88 ± 12	87 ± 21	<0.0001
GHb (%)	7.7 ± 2.8*†	4.1 ± 1	3.8 ± 0.5	<0.0001
Creatinine (mg/dl)	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.17
Fibrinogen (mg/dl)	330 ± 89*	304 ± 77	265 ± 80	0.03
Insulin (U/ml)	14.4 (4.9–51.1)*†	7.6 (2–19.7)	7.0 (1–19.8)	0.03
HOMA index	5.88 ± 3.65*	1.90 ± 1.04	1.84 ± 1.02	0.01
C-peptide (ng/ml)	2.6 (0.6–6.5)*	2.1 (0.8–5)	1.7 (0.8–3.7)	0.006
UAE (mg/g)	4.8 (3.7–16)*	4.6 (3.8–11.2)*	4.2 (3.5–16.9)	0.079
Total cholesterol (mg/dl)	232 ± 40*	271 ± 62*	161 ± 23	<0.0001
HDL (mg/dl)	44 (28–60)	47 (24–69)	48 (40–109)	0.18
LDL (mg/dl)	150 ± 46†§	200 ± 43*‡	98 ± 30	<0.0001
Triglycerides (mg/dl)	179 (133–533)*†	128 (40–260)*	75 (35–146)	<0.0001

Data are *n*, means ± SD, or medians (range). Analysis of variance was performed for comparisons among groups of normally distributed variables, and nonparametric analyses (Kruskal-Wallis) were used for insulin, C-peptide, UAE, HDL, and triglycerides. dBP, diastolic blood pressure; sBP, systolic blood pressure. *Compared with control subjects; †compared with patients with hypercholesterolemia; ‡compared with type 2 diabetic dyslipidemic patients; §*n* = 34 subjects.

terolemic nondiabetic patients, and in normal subjects. We also sought to analyze associations of these markers with clinical and laboratory variables from the study subjects.

RESEARCH DESIGN AND METHODS

Patients

Patients regularly attending the outpatient internal medicine and diabetes clinics at Hospital de Clínicas de Porto Alegre from April to October 1996 were consecutively selected. The study included patients with type 2 diabetes, as defined by the World Health Organization (WHO) (10), associated with dyslipidemia, as defined by the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (11) (total cholesterol ≥200 mg/dl, LDL cholesterol ≥160 mg/dl, HDL cholesterol <35 mg/dl, and/or triglycerides >150 mg/dl). Nondiabetic hypercholesterolemic patients, defined by presence of hypercholesterolemia and absence of diabetes, were also included. Elevated triglyceride levels and HDL levels <35 mg/dl were not required for inclusion of these patients; therefore, their lipid profile was different from that of patients with diabetes. The subjects, ages ranged from 40 to 65 years; the participants were nonsmokers (never smoked or quit over 15 years ago) and normotensive (blood pressure ≤140/90 mmHg after a 15-min rest), and they were not using any antihypertensive medication at the time of the study. Healthy volunteers without diabetes or lipid disorder, defined as total cholesterol <200 mg/dl, LDL <130 mg/dl, HDL >45 mg/dl, and triglycerides <150 mg/dl, were invited to form the control group. Albuminuria was measured in a first morning urine sample in all of the potential study participants. Patients with hypercholesterolemia and healthy control subjects were submitted to a 75-g oral glucose tolerance test to ensure their nondiabetic condition according to WHO criteria (10). All of the subjects answered the WHO cardiovascular questionnaire (12) and completed a standard questionnaire regarding current medication and known duration of diabetes. Subjects were excluded from the study in the presence of BMI >32 kg/m^2, documented renal impairment (serum creatinine >1.5 mg/dl), or an albumin-to-creatinine ratio (milligram:gram) ≥15 (13) as an estimate of urinary albumin excretion (UAE), impaired glucose tolerance according to WHO criteria (10), hypertension, history of cerebrovascular disease, previous vascular event (angina, myocardial infarction, or acute arterial occlusion), heart failure, or coexisting inflammatory diseases. Use of insulin, statins, and ACE inhibitors was also an exclusion criterion.

Study participants were divided into 3 groups as follows: patients with type 2 diabetes and dyslipidemia, patients with hypercholesterolemia, and individuals without diabetes or lipid disorders (control group). The local institutional review board and ethics committee approved the study protocol, and written informed consent was obtained from all of the individuals before enrollment.

Laboratory methods

After 12 h of fasting, blood samples were collected from all of the individuals. The plasma glucose concentration was measured by a glucose oxidase method. Total cholesterol and triglycerides were determined enzymatically (Mega–Merck; Merck, Darmstadt, Germany). HDL cholesterol was determined after VLDL and LDL precipitation with magnesium chloride and phosphotungstic acid. LDL was estimated by the Friedewald formula (14) when triglyceride levels were <400 mg/dl. Plasma and urinary creatinine levels were measured by the Jaffe reaction, and fibrinogen was assessed by immunoturbidimetry. GHb was measured by agarose gel electrophoresis (reference range 4.0–8.0%). Plasma total immunoreactive insulin levels were measured by radioimmunoassay (mean intra- and interassay coefficients of variation [CVs] 5.9 and 7.9%, respectively) (ImmuChem Coated Tube CT; ICN Pharmaceuticals, Costa Mesa, CA), and C-peptides were measured using a chemiluminescent enzyme immunoassay system (mean intra- and interassay CVs 7.2 and 7.8%, respectively) (Diagnostic Products, Los Angeles, CA). Insulin sensitivity was estimated by homeostasis model assessment (HOMA), as recently described and validated (15). Urinary albumin concentration was measured in morning sterile urine samples in duplicate by immunoturbidimetry (mean intra- and interassay CVs 10.2 and 11.8%, respectively) (MicroAlb; Bayer, Tarrytown, NY).

vWF and ET-1 measurements

Venous blood (15 ml) was drawn to an EDTA- or citrate-containing tube (for ET-1 and vWF, respectively) after a 12-h fast and a 30-min resting period in the supine position. Samples were centrifuged, and plasma was stored frozen at −70°C for subsequent

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blind analyses performed simultaneously for all of the participants. vWF was measured in duplicate samples using an enzyme-linked immunosorbent assay (ELISA) (Asserachrom vWF; Diagnostica Stago, France) (expected values were between 60 and 150% in healthy volunteers). Extraction of ET-1 was performed using a centrifugal evaporator after plasma-solvent dilution (water, hydrochloric acid, and acetone). Assays were carried out immediately after extraction. ET-1 was measured in duplicate samples by ELISA (R&D Systems, Minneapolis, MN) (sensitivity <1.0 pg/ml, mean intra- and interassay CVs 4.2 and 5.1%, respectively).

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 6.0 (SPSS, Chicago). Data with normal distribution are expressed as means ± SD or as medians and minimum-maximum intervals when the distribution is nonparametric. The χ^2 test was used for comparison of categorical variables. Analyses of variance were used for comparisons between groups of continuous variables. Nonparametric analysis of variance (Kruskal-Wallis) was applied for skewed variables. Pairwise comparisons between groups were performed using the Student-Newman-Keuls test. Multiple linear regression was used to analyze factors that were independently associated with ET-1 and vWF levels. First, models were built to evaluate whether disease status (type 2 diabetes with dyslipidemia and hypercholesterolemia) explained changes in ET-1 and vWF after adjustment for differences among groups. Variables showing differences with a P value <0.10 in the univariate analysis were included in the models (backward elimination). Second, data were analyzed regardless of disease status to determine clinical and laboratory variables associated with ET-1 and vWF levels. A 2-tailed P value <0.05 was considered significant.

RESULTS

Characteristics of the patients

Of the 99 individuals who were initially evaluated, only 75 were enrolled as study subjects because 13 individuals were excluded on the basis of impaired glucose tolerance, and 11 were excluded on the basis of inadequate lipid profile. Thus, 35 patients with type 2 diabetes plus dyslipidemia, 21 nondiabetic patients with hypercholesterolemia, and 19 healthy control subjects were studied. No differences in sex distribution were observed between the groups. The mean known duration of diabetes was 6.3 years, ranging from 5 months to 14 years (1 patient). Diabetic patients were treated by diet alone or diet plus sulfonylurea preparations. No patients were using biguanide. Patients with type 2 diabetes plus dyslipidemia were older and had a higher systolic blood pressure than individuals in the other groups. BMI was lower in control subjects. As expected, fasting insulin ($P < 0.03$) and C-peptide levels ($P < 0.006$) were significantly higher in patients with type 2 diabetes and dyslipidemia. Fibrinogen levels were significantly higher in patients with diabetes plus dyslipidemia when compared with control subjects ($P < 0.03$) but not when compared with hypercholesterolemic patients. Except for HDL levels, all of the variables—glucose, GHb, and lipid profile—differed between the 3 groups (Table 1).

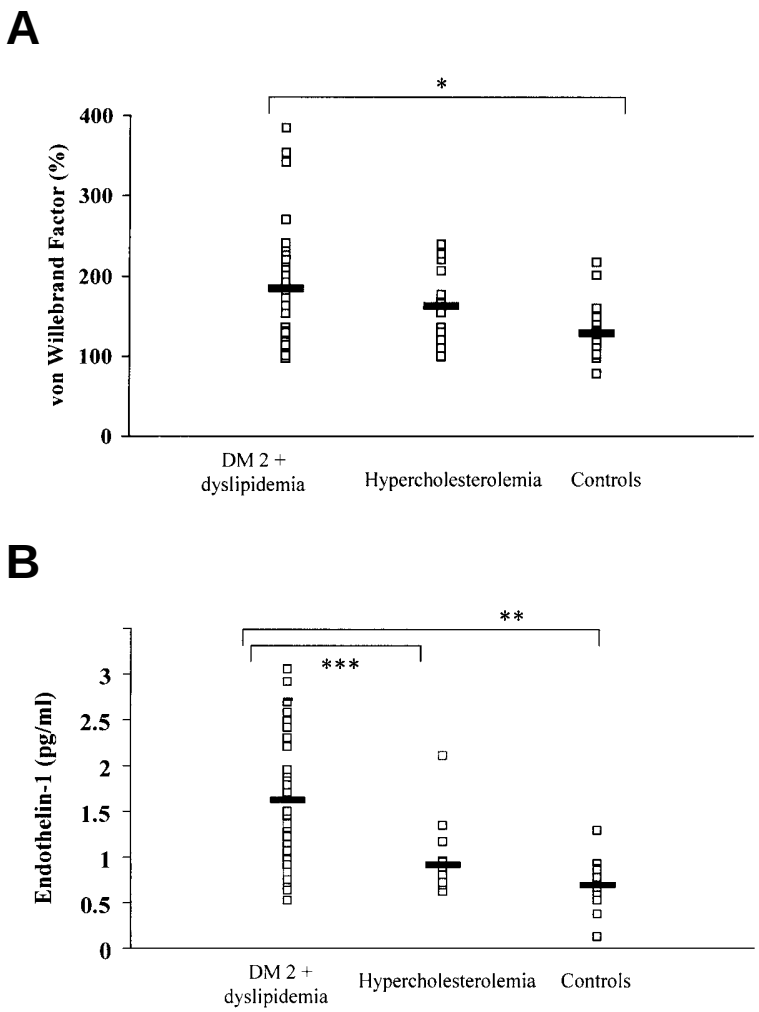

Figure 1—A*: Individual vWF plasma levels in the 3 study groups.* B*: Individual ET-1 plasma levels in the 3 study groups. Horizontal black lines indicate mean values. DM 2, type 2 diabetes.* *P = *0.005;* **P = *0.0001;* ***P = *0.0002.*

Endothelial markers

vWF levels were significantly higher in patients with diabetes and dyslipidemia (185.49 ± 72.1%) when compared with control subjects (129.70 ± 35.2%, $P = 0.02$). vWF levels were similar in nondiabetic patients with hypercholesterolemia and control subjects (163.29 ± 50.7%) (Fig. 1*A*). ET-1 levels in turn were significantly increased ($P < 0.0001$) in patients with type 2 diabetes and dyslipidemia (1.62 ± 0.73 pg/ml) when compared with patients with hypercholesterolemia (0.911

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Table 2—*Multivariate analysis of ET-1 and vWF*

Models	β coefficient ± SEM	*P*	Adjusted r^2
With disease status			
ET-1			0.47
Type 2 diabetes and dyslipidemia	0.38 ± 0.18	0.04	
Triglyceride levels (mg/dl)	0.002 ± 0.0008	0.03	
UAE (mg/g)	0.03 ± 0.02	0.11	
vWF			0.22
Type 2 diabetes and dyslipidemia	24 ± 20	0.22	
UAE (mg/g)	7.7 ± 2.4	0.0025	
Without disease status			
ET-1			0.45
Triglyceride levels (mg/dl)	0.0025 ± 0.0008	0.002	
Age	0.019 ± 0.009	0.04	
HOMA	0.20 ± 0.08	0.009	
UAE (mg/g)	0.044 ± 0.021	0.03	
vWF			0.22
UAE (mg/g)	7.7 ± 2.2	0.001	
Fibrinogen (mg/dl)	0.17 ± 0.07	0.02	
BMI (kg/m^2)	4.0 ± 1.8	0.03	

Data are adjusted for age, BMI, systolic blood pressure, fibrinogen, HOMA, and LDL levels.

± 0.73 pg/ml) and control subjects (0.69 ± 0.25 pg/ml) (Fig. 1*B*).

Multiple linear regression analysis

Plasma ET-1 was significantly associated with the presence of type 2 diabetes plus dyslipidemia, triglyceride levels, and UAE after adjustment for age, BMI, systolic blood pressure, fibrinogen, HOMA, and LDL levels. For vWF values, the same procedure was performed, and after adjustment for differences among groups, disease status did not have a significant impact on this variable. UAE was the only clinical factor that contributed to vWF profile in this analysis (Table 2).

When ET-1 and vWF levels were separately analyzed as dependent variables, irrespective of the patient group (models without disease status), age, triglyceride, HOMA, and UAE significantly suggested higher ET-1 levels. vWF levels were significantly associated with UAE, fibrinogen levels, and BMI (Table 2).

CONCLUSIONS — This sample of normotensive normoalbuminuric type 2 diabetic patients with dyslipidemia presented increased levels of plasma ET-1 and vWF compared with normal individuals. These abnormalities were significantly associated with UAE levels within the normal range. Moreover, increased plasma levels of ET-1, irrespective of the study group, were also associated with features of the insulin resistance syndrome, such as triglyceride levels and the HOMA index. In contrast to ET-1, vWF was not associated with type 2 diabetic dyslipidemia after adjustment for differences between the groups, but it did appear to be influenced by UAE levels.

The observation of increased levels of ET-1 and vWF in normoalbuminuric normotensive type 2 diabetic patients with dyslipidemia is in accordance with previous findings showing impaired endothelium-dependent vasodilatation and elevated ET-1 values in subjects with type 2 diabetes (6,16). However, unlike most previous reports, patients included in this study did not have diabetic complications or any other apparent risk factor for endothelial damage apart from diabetes and dyslipidemia or hypercholesterolemia. Even though the selection process intended to include only patients with these characteristics, higher levels of systolic blood pressure, although within the normal range, and higher BMI were observed in patients with diabetes, which probably reflects phenotypic characteristics of the insulin resistance syndrome. Triglyceride levels were particularly elevated in type 2 diabetic patients, which is in accordance with the presence of typical diabetes and insulin resistance syndrome–associated dyslipidemia. Although nondiabetic patients with hypercholesterolemia clearly had a severely abnormal lipid profile, neither their ET-1 nor their vWF levels were different from those of control subjects. These 2 types of lipid disorders, which are associated with triglyceride levels in diabetic and with hypercholesterolemia in nondiabetic patients, could have a different impact on the vascular endothelium.

The association between diabetes and dyslipidemia determined significant differences, especially on ET-1 levels. In the multivariate analysis, only triglycerides, as a lipid element, appeared to have contributed to this finding. However, qualitative oxidative changes in LDL may be as important as absolute quantitative values in causing injury to the endothelium (17). It is known that in diabetes, LDL particles undergo qualitative changes and become more atherogenic; in addition, an increase in the turnover of triglyceride-rich lipoproteins in diabetes could generate particles that augment the risk of atherosclerosis (18). Thus, it is likely that the clustering of different metabolic abnormalities and the sum of those pertaining to the diabetic state and to dyslipidemia may explain, at least partially, the differences in the ET-1 and vWF values observed. In fact, only ET-1 was significantly higher in patients with type 2 diabetes and dyslipidemia compared with hypercholesterolemic patients. This result may indicate that ET-1 might be a more sensitive marker than vWF in this setting. Nonetheless, these observations support the notion that features of the insulin resistance syndrome may also influence vWF behavior and that increased thrombogenecity may be signaled by vWF because fibrinogen is associated with vWF levels.

Endothelial dysfunction may precede the development of atherosclerosis in the presence of different risk factor conditions (19). The increasing knowledge regarding how and how soon the endothelium can be injured, the occurrence of which produces a large variety of biologic markers, has recently led to the assumption that these markers can be a surrogate for endothelial dysfunction. In the present study, patients without any clinically apparent cardiovascular disease or other known risk factors for atherosclerosis, except for type 2 diabetes and dyslipidemia, showed different degrees of endothelial dysfunction as assessed by ET-1 and vWF levels. Several metabolic abnormalities present in diabetes and dyslipidemia could account for endothelial damage. In this study, ET-1 and vWF were significantly associated with HOMA. HOMA

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is a mathematical model that estimates insulin sensitivity based on fasting plasma glucose and insulin concentrations (15). Other authors have already reported an association between endothelial dysfunction and features of the insulin resistance syndrome (9,20). Endothelial dysfunction has been associated with high insulin levels and obesity, independently of hypertension, hypercholesterolemia, or age, even in nondiabetic subjects (21). There is evidence that insulin stimulates nitric oxide production through the insulin receptor. Lack of insulin or resistance to its actions may depress nitric oxide production (22).

The fact that UAE levels were associated with both markers, ET-1 and vWF, underscores the concept that microalbuminuria is a marker of widespread vascular damage (23) and an independent risk factor for coronary artery disease (24). Increased ET-1 levels were reported in microalbuminuric type 2 diabetic patients (25) and in type 2 microalbuminuric diabetic patients with cardiovascular disease (20). Stehower et al. (26) observed that endothelial dysfunction, estimated by plasma vWF, may predict the development of microalbuminuria in type 1 diabetic patients. Our observations add novel information to this field, because in our study UAE levels were within normal range, i.e., patients were assessed before the development of microalbuminuria. However, no association between UAE and ET-1 was observed when type 2 diabetes was included in the regression models, perhaps because the diabetic state clusters powerful components that are involved in endothelial dysfunction and thereby reduces the impact of each element individually.

Possible limitations of this study are related to fundamental differences between the groups, such as increased levels of systolic blood pressure (although within the normal range), age, and BMI. This is conceivable because of the entry criteria; for example, it was extremely difficult to identify older control subjects who fulfilled all of the criteria of a normal metabolic profile. Although these variables were included in the multivariate analyses, they may have influenced the results because statistical models only partially correct for design disparities. In addition, it was not possible to characterize the role of the diabetic state alone in causing endothelial dysfunction. It would be interesting to further explore this issue; however, this scenario may be of limited clinical value because diabetic patients without lipid abnormalities are rare.

In conclusion, the present study demonstrates that normotensive normoalbuminuric patients with type 2 diabetes plus dyslipidemia have higher levels of ET-1 and vWF compared with nondiabetic glucose-tolerant hypercholesterolemic subjects and healthy control subjects, respectively. The behavior of these markers, especially ET-1, appeared to be influenced by UAE and other metabolic and phenotypic changes compatible with the insulin resistance syndrome.

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