Advanced Glycation End Products and Antioxidant Status in Type 2 Diabetic Patients With and Without Peripheral Artery Disease

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OBJECTIVE — Advanced glycation end products (AGEs), pentosidine and malondialdehyde (MDA), are elevated in type 2 diabetic subjects with coronary and carotid angiopathy. We investigated the relationship of AGEs, MDA, total reactive antioxidant potentials (TRAPs), and vitamin E in type 2 diabetic patients with and without peripheral artery disease (PAD).

RESEARCH DESIGN AND METHODS — AGEs, pentosidine, MDA, TRAP, vitamin E, and ankle-brachial index (ABI) were measured in 99 consecutive type 2 diabetic subjects and 20 control subjects.

RESULTS — AGEs, pentosidine, and MDA were higher and vitamin E and TRAP were lower in patients with PAD (ABI <0.9) than in patients without PAD (ABI >0.9) ($P < 0.001$). After multiple regression analysis, a correlation between AGEs and pentosidine, as independent variables, and ABI, as the dependent variable, was found in both patients with and without PAD ($r = 0.9198$, $P < 0.001$ and $r = 0.5764$, $P < 0.001$, respectively) but not in control subjects. When individual regression coefficients were evaluated, only that due to pentosidine was confirmed as significant. For patients with PAD, considering TRAP, vitamin E, and MDA as independent variables and ABI as the dependent variable produced an overall significant regression ($r = 0.6913$, $P < 0.001$). The regression coefficients for TRAP and vitamin E were not significant, indicating that the model is best explained by a single linear regression between MDA and ABI. These findings were also confirmed by principal component analysis.

CONCLUSIONS — Results show that pentosidine and MDA are strongly associated with PAD in type 2 diabetic patients.

Diabetes Care 30:670–676, 2007

Diabetes is associated with a greatly increased risk of cardiovascular disease, which cannot be explained by known risk factors, such as smoking, hypertension, and dyslipidemia (1).

Recent studies indicate that hyperglycemia is an important contributor to the development of these complications. In this framework, among the biochemical alterations characteristic of hyperglycemia, factors involved in determining atherosclerotic disease are formation of advanced glycation end products (AGEs), increased polyol pathway flux, increased hexosamine pathway flux, and protein kinase C activation (2–5). All of these molecular mechanisms reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain. Thus, hyperglycemia and increased oxidative stress (6) lead to tissue damage through common pathways. In particular, AGEs may cause damage through the formation of abnormal cross-links in collagen, thus contributing to vascular stiffening (2–5); modification of lipoprotein, as a result of glycation, may contribute to foam cell formation (2–5). In diabetes, reduced antioxidant defenses have also been described, thus providing an additional contribution to the development of chronic complications (7).

Recent studies reported that the serum level of AGEs is increased in type 2 diabetic patients with coronary heart disease (8–10). Immunohistochemical studies have also shown that AGEs accumulate in coronary atherosclerotic plaques and cardiac tissue of diabetic patients (11). Among AGEs in patients with type 2 diabetes, high serum pentosidine (a marker of glycoxidation-induced cross-linking) is associated with both increased carotid intima-media wall thickness and arterial stiffening (12). Malondialdehyde (MDA) is frequently measured as an indicator of lipid peroxidation and oxidative stress in vivo and is elevated in diabetic patients with macroangiopathy (13). Peripheral artery disease (PAD) is a strong predictor of coronary and carotid atherosclerosis (14–16) and affects about 29% of patients with type 2 diabetes (17).

To our knowledge, no data are available about the involvement of AGEs and oxidative stress in PAD in diabetic patients. The aim of our study was to detect a value of the ankle-brachial index (ABI) of <0.9, indicating the presence of PAD, in a group of type 2 diabetic patients and to compare those with positive results with those with negative results with respect to glycolipid oxidation products

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Received for publication 18 July 2006 and accepted in revised form 28 November 2006.

Abbreviations: ABI, ankle-brachial index; AGE, advanced glycation end product; FPG, fasting plasma glucose; LC, liquid chromatography; MDA, malondialdehyde; PAD, peripheral artery disease; PCA, principal component analysis; R-PE, R-phycoerythrin; TRAP, total reactive antioxidant potential.

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

DOI: 10.2337/dc06-1508

and antioxidant defenses as total reactive antioxidant potentials (TRAPs) and vitamin E.

RESEARCH DESIGN AND METHODS

— For our study, we evaluated blood levels of AGEs, pentosidine, MDA, TRAP, and vitamin E in a group of type 2 diabetic patients with and without PAD. Ninety-nine consecutive type 2 diabetic patients (57 men and 42 women) who regularly attended our outpatient clinic were enrolled in the study. Coronary heart disease was evaluated by clinical history (specified from charts) of myocardial infarction, angina pectoris, coronary artery surgery, angioplasty, and/or definite myocardial infarction on an electrocardiogram, interpreted according to the Minnesota code (18). Cerebral vasculopathy was investigated by history of symptoms of transient ischemic attack and/or stroke. Hypertension was diagnosed if blood pressure was >130/80 mmHg and/or if antihypertensive drugs were being taken (19). A physical examination and ophthalmoscopy were also performed.

The control group was composed of 20 subjects (10 men and 10 women) who were selected from an initial group of 32 subjects with normal glucose tolerance according to the American Diabetes Data Group criteria (20). Compared with the diabetic patients, the control subjects had similar distributions of sex, age, and diet, particularly as regards consumption of fruit and fresh vegetables. Informed written consent was obtained from all subjects participating in the study.

No diabetic patients were receiving insulin therapy, but >90% of them took both hypoglycemic and antihypertensive drugs. Thirty percent of patients with PAD and 27% without PAD took statins; 91% of patients with PAD and 45% without ($P < 0.001$) were receiving antiplatelet therapy (aspirin 100 mg/day).

On the day of the study, fasting blood samples were taken to determine fasting plasma glucose (FPG), A1C, pentosidine, AGEs, TRAP, vitamin E, MDA, cholesterol, and triglycerides. In the morning, after a 12-h overnight fast, blood was collected and immediately centrifuged at 1700*g* for 20 min at 4°C. Glucose was measured immediately, and the remaining samples were frozen at −80°C until assayed. The assays were performed within 3 months of sample collection. Under these conditions, previous experience had proven that no alteration occurs.

The ABI was estimated at rest by strain-gauge plethysmography, by means of a compressor connected to three cuffs, placed around one arm (that with higher pressure) and both ankles; distally, mercury strain gauges were placed, respectively, around the thumb of the abovementioned arm and both terminal digits (toes) of the foot. By inflating the cuff to a pressure sufficient to stop blood flow, the plethysmographic transducer reveals a flat line. During slow deflation of the cuff, the appearance of the wave pulse indicates the level of systolic pressure of that area. The ABI is the ratio between the systolic pressures of ankles and arm, measured at the same time (21). A value of <0.9 was considered to indicate PAD, according to the conventional cutoff (15,16). The lowest mean ABI of three consecutive measurements in both legs was used in analysis. All subjects were also evaluated by echo color Doppler ultrasound of the peripheral arterial hypoaortic tree.

Pentosidine was measured by a liquid chromatography (LC) method (22). Plasma samples (350 μl), after the addition of 100 μl of $NaBH_4$ (0.2 mol/l), were precipitated on ice with an equal volume of 10% cold trichloroacetic acid. Samples were then hydrolyzed (6 N HCl at 110°C for 18 h), and, after acid removal (centrifugation with a Speed Vac SC 110; Savant), pellets were suspended in 500 μl of 0.01% heptafluorobutyric acid (Sigma-Aldrich, Milan, Italy) and then filtered. Samples were analyzed by LC (ProStar detector; Varian, Turin, Italy). The column was a 3.9 × 300 mm C_{18} μBondapack (Waters Italia, Milan, Italy). The LC equipment was programmed with a linear gradient of acetonitrile in water and 0.1% heptafluorobutyric acid. Pentosidine was detected by fluorescence excitation at 335 nm and emission at 385 nm. The pentosidine standard was a kind gift of Professor V.M. Monnier (Case Western Reserve University, Cleveland, OH).

AGEs were measured by an enzyme-linked immunosorbent assay method (23), using AGE-BSA as the adsorbed antigen. AGE-albumin was prepared by incubating albumin (50 mg) with 0.5 mmol/l glucose in 0.2 mol/l $NaPO_4$ buffer (pH 7.4) under sterile condition. After incubation, unbound material was removed by extensive dialysis against PBS. Microliter plates were coated with AGE-BSA by adding 100 μl of a solution of AGE-BSA (30 μl/ml dissolved in PBS) to each well and incubating for 2 h at room temperature. Wells were washed three times with 0.15 ml of a solution containing PBS, 0.05% polyoxyethylene sorbitol ester (Tween 20) (PBS-Tween; Sigma Chemical, St. Louis, MO), and 1 mmol/l NaN_3. Wells were then blocked by incubation for 1 h with 0.1 ml of a solution of blocking buffer (a proprietary protein in PBS containing Kathon Antimicrobial Agent; Pierce Biotechnology, Rockford, IL). After washing with PBS-Tween, 50 μl of competing antigen was added, followed by 50 μl of antiserum. Plates were incubated for 3 h at room temperature. Wells were then washed with PBS-Tween and developed with alkaline phosphatase-linked anti-rabbit IgG (an antibody directed against rabbit IgG, purified from serum by chromatography after immunization of sheep with rabbit IgG; Roche Diagnostics, Mannheim, Germany), with *p*-nitrophenyl phosphate as the colorimetric substrate.

TRAP was evaluated according to the method of Ghiselli et al. (24). In this method, the production of peroxyl radicals obtained by thermal decomposition of 2,2′-azobis-(2-amidinopropane) dihydrochloride leads to a linear decrease in R-phycoerythrin (R-PE) fluorescence emission over 1 h. When plasma is added to the reaction mixture, a period of complete protection of R-PE is observed. The length of this lag phase (*T*) is here taken to be directly related to total plasma antioxidant capacity. To quantify TRAP, the *T* produced by plasma is compared with the *T* produced by a known amount of Trolox. By comparing the *T* of plasma with the *T* of Trolox, taking into account the concentration of Trolox, the TRAP value of a plasma sample is obtained according to the following proportion: concentration Trolox:*T* Trolox = *X*:*T* plasma.

The resulting value of *X* is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by the dilution factor of plasma (250); values are expressed as micromoles per liter. 2,2′-Azobis-(2-amidinopropane) dihydrochloride, R-PE, Trolox, and all other chemicals were purchased from Sigma Chemical.

Vitamin E levels were measured by high-precision chromatography on a reverse-phase column by a diode array spectrophotometric detector, according to the method of Tsan et al. (25). MDA was measured by the highly sensitive fluorometric method (high-performance LC) after deproteination of the sample and its reaction with thiobarbituric acid and extraction of the adduct with *n*-butylic alcohol (26). Plasma glucose was

Table 1—*Clinical characteristics and laboratory and instrumental data of patients and healthy subjects*

Parameters	Type 2 diabetic patients with PAD	Type 2 diabetic patients without PAD	Healthy subjects
n	33	66	20
Age (years)	64.7 ± 4.6	64.1 ± 6.2	62.1 ± 4.1
Sex (male/female)	19/14	39/27	10/10
Diabetes duration (years)	8.8 ± 6.4	8.3 ± 5.3	—
BMI (kg/m^2)	27.9 ± 4.8	28.9 ± 4.1*	25.4 ± 3.5
Fasting plasma glucose (mg/dl)	167.0 ± 54.1	158.5 ± 43.5†	89.2 ± 4.4‡
A1C (%)	8.12 ± 1.50	7.57 ± 1.27†	5.56 ± 0.32‡
Total serum cholesterol (mg/dl)	220.7 ± 35.1	220.4 ± 31.1	212.0 ± 30.0
LDL cholesterol (mg/dl)	132.5 ± 37.4	140.6 ± 29.8	135.0 ± 36.0
HDL cholesterol (mg/dl)	51.5 ± 10.9	50.5 ± 11.6	53.0 ± 15.0
Triglycerides (mg/dl)	155.7 ± 63.3	147.0 ± 77.0	110.0 ± 45.0
AGEs (μg/mg protein)	14.8 ± 5.5§	10.9 ± 3.5*	7.3 ± 2.4‡
Pentosidine (pmol/ml)	109.2 ± 24.4§	84.5 ± 19.8†	63.2 ± 7.7‡
Malondialdehyde (μmol/l)	1.29 ± 0.39§	0.99 ± 0.29	0.80 ± 0.20‡
Vitamin E (μmol/l)	7.34 ± 2.00‖	9.07 ± 2.04†	11.5 ± 3.6‡
TRAP (μmol/l)	746.4 ± 119.9§	828.8 ± 90.9†	962.0 ± 71.6‡
Serum creatinine (mg/dl)	0.89 ± 0.06	0.86 ± 0.07	0.87 ± 0.02
ABI	0.69 ± 0.13§	1.07 ± 0.11	1.08 ± 0.08‡
Coronary heart disease	7 (21)	8 (12)	0¶
Cerebrovascular events (transient ischemic attack/stroke) (*n*)	0	0	0
Smokers	8 (24)	15 (23)	4 (20)

Data are means ± SD or *n* (%) unless otherwised indicated. One-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons was used for continuous variables; χ^2 test with Yates' correction or Fisher's exact test (for small values, <5) was used for frequency data. *$P < 0.01$, †$P < 0.001$, patients without PAD vs. healthy subjects; ‡$P < 0.001$, patients with PAD vs. healthy subjects; §$P < 0.001$, ‖$P < 0.01$, patients with PAD vs. patients without PAD; ¶$P < 0.05$, patients with PAD vs. healthy subjects.

determined by a glucose oxidase method (27). A1C was measured by an LC method (28) (Bio-Rad, Milan, Italy). Total cholesterol and LDL and HDL cholesterol were measured by enzymatic analytical chemistry (CHOD-PAP method, Roche Diagnostics, Milan, Italy) (29,30) as plasma triglycerides (GPO-PAP colorimetric enzyme test, Roche) (31).

Statistical analysis

Data are expressed as means ± SD. For parameters occurring as frequency, the statistical difference between the two groups of patients was determined by means of the χ^2 test with Yates' correction (32); when a frequency was <5, Fisher's exact test was applied.

For statistical multiple comparison of continuous variables, a one-way ANOVA followed by the Bonferroni post hoc test was used. Statistical difference was accepted when $P < 0.05$.

Linear regression. The presence of any relationship between pair variables was evaluated by least-squares linear regression. Pearson's correlation coefficient *r* was used to quantify the strength of the relationship.

Multiple regression. Multiple regression was used to further explore the linear relationship among the variables. The equation was of the form: $y = a + b_1x_1 + b_2x_2 + b_3x_3$.

Regression parameters were estimated as well as correlation coefficient *r*. ANOVA statistics were used to assess the significance of the regression and accepted for $P < 0.05$.

Principal component analysis. Principal component analysis (PCA) is useful in reducing the dimensionality of the dataset, may help to identify new meaningful underlying variables, and can also indicate the presence of clusters within multivariate data. PCA transforms a number of possibly correlated variables into fewer uncorrelated variables defined as principal components, which are linear combinations of the original variables. After principal components were obtained, they were plotted to observe any groupings in the dataset. PCA computation was performed according to a correlation matrix and standardized principal component score using AMADA software (33) or BiPlot software Excel macros (34). The first three components were considered for classification of data. A BiPlot graphic display was used to present the behavior of variables (columns) to examine their correlation (35) on the same chart. Both length and directions of vectors (rays) may be important in interpreting the data (35). In this case, the most useful parameter is the cosine of the eigenvectors, which suggested correlations among different variables. When the angle between eigenvectors is close to 0°, the variables are positively correlated, the angle for negative correlations approaches 180°, and angles of 90° indicate no correlation.

RESULTS

— On the basis of the ABI values, the 99 type 2 diabetic patients were divided into one subgroup of 33 individuals with PAD (ABI ≤0.9) and another subgroup of the remaining 66 patients without PAD (ABI >0.9). All of the diabetic patients with PAD showed the characteristic below-the-knee macroangiopathy, without media calcification, confirming ABI data obtained by plethysmography. No evidence of macroangiopathy above the knee was found.

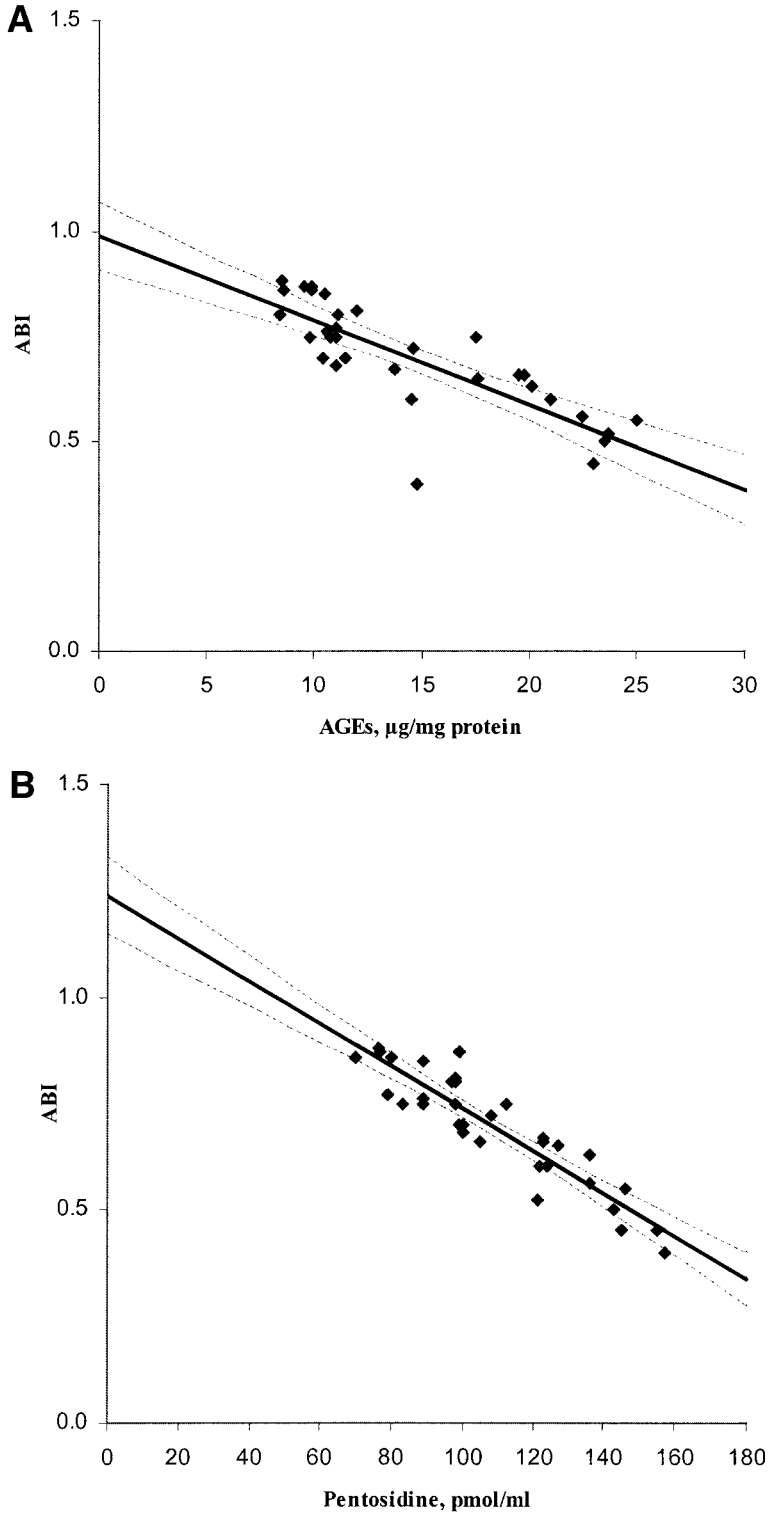

Figure 1—*Correlations between ABI and serum AGEs* (A) *and between ABI and serum pentosidine* (B) *in diabetic patients with PAD.* y = *−0.0201*x + *0.9887;* r^2 = *0.662;* r = *0.8162;* P < *0.001.* – – –, *95% CI.*

The clinical characteristics and laboratory and instrument data of patients and control subjects are listed in Table 1. No significant differences were found in age or BMI between type 2 diabetic patients with or without PAD nor was there any difference in diabetes duration or smoking habits.

There were no significant differences in serum levels of A1C, FPG, or lipid profiles. Serum creatinine values were normal in all subjects, and none of the diabetic patients had retinopathy. Serum concentrations of AGEs, pentosidine, and MDA were significantly higher in patients with vasculopathy than in patients without ($P < 0.001$ for each parameter). Vitamin E and TRAP were significantly lower in patients with PAD ($P < 0.001$).

When attempting to fit data pairwise using linear regression analysis and considering all diabetic patients, we found no significant correlations between FPG or A1C against any glycolipid oxidation parameter evaluated (AGEs, pentosidine, and MDA) and ABI. Furthermore, no correlations were found between either cholesterol (total, LDL, and HDL) or triglyceride levels and ABI.

After data were fitted using the linear regression model, ABI was inversely correlated with serum AGE concentrations in all patients (slope = −0.0308, r = −0.6685, $P < 0.001$), even when patients with PAD (slope = −0.0201, r = −0.8162, $P < 0.001$) (Fig. 1*A*) and without PAD (slope = −0.0166, r = −0.5366, $P < 0.001$) were considered. ABI was also found to be correlated with pentosidine in all patients (slope = −0.0065, r = −0.7490, $P < 0.001$) and when the groups with PAD (slope = −0.0050, r = −0.9154, $P < 0.001$) (Fig. 1*B*) and without PAD were evaluated separately (slope = −0.0031, r = −0.5716, $P < 0.001$). A negative relationship was also found between ABI and MDA but was significant only in patients with PAD (slope = −0.2292, r = −0.6714, $P <$ 0.001). In patients with PAD, both TRAP and vitamin E were directly associated with ABI (slope = 0.0005, r = 0.4318, $P < 0.05$ and slope = 0.0365, r = 0.5445, $P < 0.01$, respectively).

A multiple regression model applied to AGEs and pentosidine, as independent variables, and ABI, as a dependent variable, revealed a significant correlation in both patients with and without PAD (r = 0.9198, $P < 0.001$ and r = 0.5764, $P <$ 0.001, respectively). However, no significance was found when this model was applied to healthy control subjects. When individual regression coefficients (slopes) were tested for significance by ANOVA, the component related to pentosidine was confirmed to be significant (slope = −0.00426, $P < 0.001$ for patients with PAD; slope = −0.00239, $P < 0.05$ for those without PAD). The AGE component did not reach a statistically signifi-

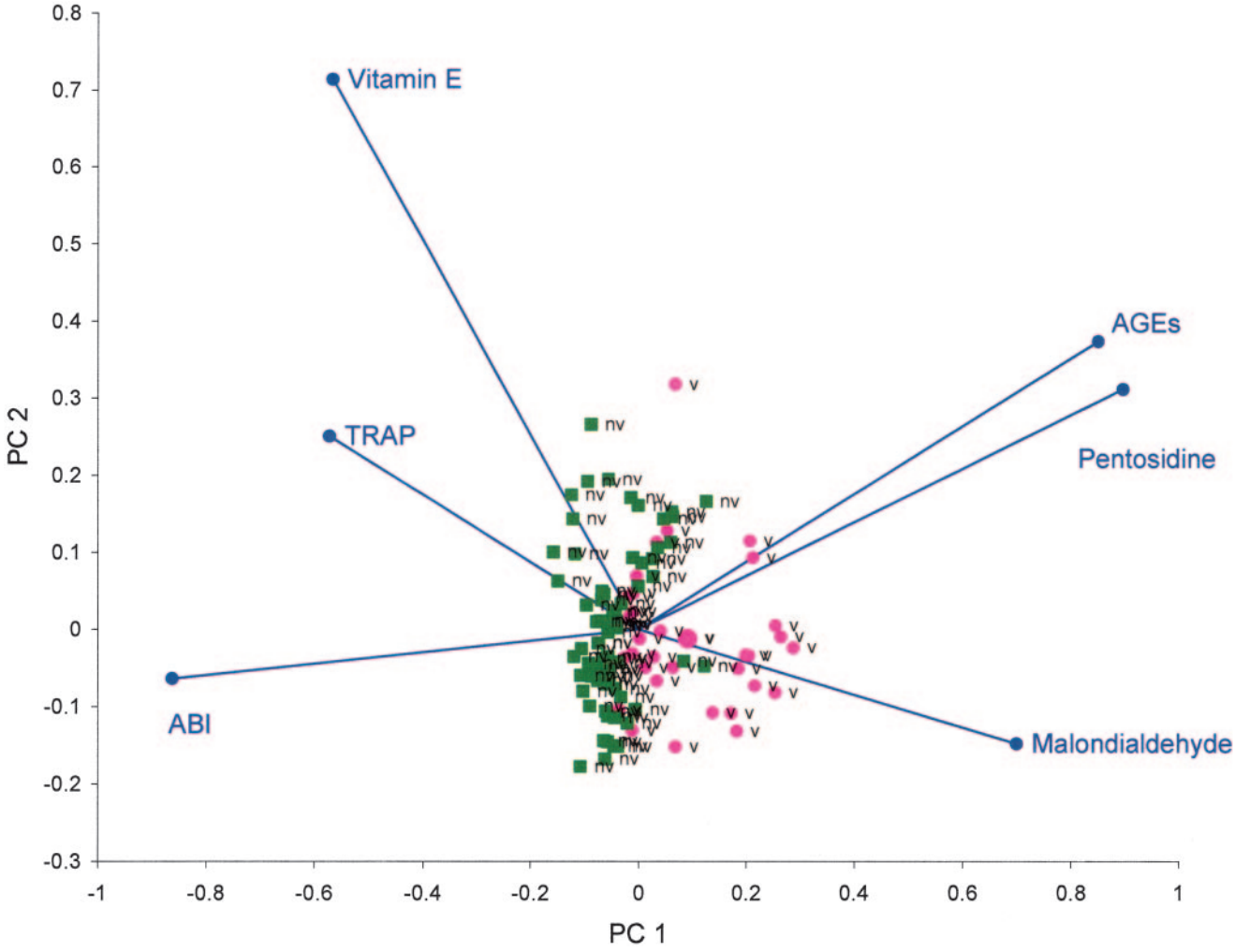

Figure 2—*BiPlot of first two principal components obtained with PCA analysis (columns centered and standardized, BiPlot software) conducted on six among most representative variables from all patients (v, vasculopathic, with PAD; nv, not vasculopathic, without PAD).*

cant level, indicating that the model is not closely dependent on the latter variable.

Multiple regression was also performed for TRAP, vitamin E, and MDA to verify any link with the dependent variable ABI. In this case, for patients with PAD, a significant regression was obtained ($r = 0.6913$, $P < 0.001$), but the regression coefficient due to TRAP and vitamin E was not significant, indicating that the model is best explained by a single linear regression between MDA and ABI.

A further attempt to explain the links among the variables was to use the multivariate technique of PCA applied to the most important variables considered previously. Both positive and negative correlations among variables after PCA are easily observed when the results are presented as a BiPlot graph (Fig. 2), as indicated under RESEARCH DESIGN AND METHODS. The scores obtained for the two groups of patients, although closely related, show different behavior, revealing the distinct pattern due to PAD. Considering the relationships among variables, the BiPlot graph obtained with the first PCA components shows that there is close collinear behavior between AGEs and pentosidine and that they are inversely correlated with ABI. TRAP and MDA, respectively, also appear to be inversely correlated. MDA is negatively correlated with vitamin E and TRAP. Conversely, no apparent relation was found for either AGEs or pentosidine against vitamin E.

CONCLUSIONS — In 99 consecutive type 2 diabetic patients, highly significant increased levels of AGEs, pentosidine, and MDA were found in patients with PAD more than in patients without PAD and control subjects. AGEs and pentosidine were correlated with ABI in all diabetic patients, not only in patients with PAD; no correlation was found in the control group. These results indicate that glyco-oxidation contributes to the development of atherosclerosis in the below-the-knee peripheral artery tree in type 2 diabetes. More precisely, among AGE components, pentosidine appears to be strongly associated with the peripheral artery status of diabetic patients. This study indicates that pentosidine may be a predictor of PAD in diabetes, but it is necessary to carry out an appropriately designed longitudinal study to confirm this hypothesis. In type 2 diabetes, previous studies have demonstrated both increased serum levels of AGEs in patients with coronary artery disease (9) and increased accumulation of AGEs in atherosclerotic plaques in coronary arteries (11). Yoshida et al. (12) also showed that accumulation of pentosidine in the vessel walls of diabetic patients increases arterial stiffness and/or carotid intima-media

thickness, contributing to their increased cardiovascular risk. For the first time, our results also show that the lower extremity arteries may be damaged by AGEs, particularly pentosidine, in type 2 diabetes. In addition, lipid oxidation, in terms of serum levels of MDA, was associated with peripheral diabetic angiopathy, in agreement with one of our previous studies (36).

We found that both TRAP and vitamin E levels, as expressions of a defense mechanism against glycolipid oxidation, were lower in type 2 diabetic patients with PAD than in those without PAD and control subjects. Low TRAP levels have been reported in both type 1 and type 2 diabetes (7,24). Recent epidemiological studies suggested that decreased levels of antioxidants favor cardiovascular disease in nondiabetic subjects (37–39). Several studies later addressed the issue of antioxidant status in type 2 diabetes, generating conflicting results (40,41). In our study, both these parameters were positively correlated with ABI in patients with PAD (lower values of ABI corresponded to lower values of TRAP and vitamin E). Moreover, these parameters were not correlated with AGEs or pentosidine. Only TRAP was inversely correlated with MDA. This suggests that the defensive role of antioxidants fails in the presence of the already developed vascular damage in type 2 diabetic patients. These findings may help to explain the inefficacy of treatment with antioxidants used in secondary prevention when the atherosclerotic process is already manifest. Several randomized trials have failed to show any benefit deriving from the use of vitamin E in preventing cardiovascular events in various high-risk groups, including diabetic patients (42,43). The Primary Prevention Project (PPP) trial (44) also showed the substantial failure of antioxidant vitamin E supplementation in primary prevention of major cardiovascular events in patients at risk. In that study, a marginal reduction in the risk of PAD was documented only in nondiabetic subjects.

It is possible that vitamin E supplementation had no effect at all. Alternatively, the lack of effect in those diabetic groups may be due to the fact that the study did not specify the duration of diabetes, although it is known (45) that PAD may occur even in patients with newly diagnosed diabetes and that it increases with the duration of disease. In addition, PAD is frequently asymptomatic in diabetic subjects.

In summary, our results show that, in type 2 diabetic patients, glyco-oxidation, lipid oxidation, and specifically serum levels of pentosidine and MDA are strongly associated with below-the-knee diabetic macroangiopathy. Serum antioxidant capacity, represented by TRAP, is associated with MDA but not with AGEs, showing that it cannot prevent the development of PAD caused by AGEs.

References

1. Kannel WB, McGee DL: Diabetes and cardiovascular disease: the Framingham study. *JAMA* 241:2035–2038, 1979
2. Vlassara H, Palace MR: Diabetes and advanced glycation end products. *J Intern Med* 251:87–101, 2002
3. Yan SF, Ramasamy R, Naka Y, Schmidt AM: Glycation, inflammation and RAGE: a scaffold for the macrovascular complications of diabetes and beyond. *Circ Res* 93:1159–1169, 2003
4. Basta G, Schmidt AM, De Caterina R: Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res* 63:582–592, 2004
5. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 4:813–820, 2001
6. Giuliano D, Ceriello A, Paolisso G: Oxidative stress and diabetic vascular complications. *Diabetes Care* 19:257–267, 1996
7. Ceriello A, Bortolotti N, Falleti E, Taboga C, Tonutti L, Crescentini A, Motz E, Lizzio S, Russo A, Bartoli E: Total radical-trapping antioxidant parameter in NIDDM patients. *Diabetes Care* 20:194–197, 1997
8. Ono Y, Aoki S, Ohnishi K, Yasuda T, Kawano K, Tsukada Y: Increased serum levels of advanced glycation end-products and diabetic complications. *Diabetes Res Clin Pract* 41:131–137, 1999
9. Kilhovd BK, Berg TJ, Birkeland KI, Thorsby P, Hanssen KF: Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care* 22:1543–1548, 1999
10. Aso Y, Inukai T, Tayama K, Takemura Y: Serum concentrations of advanced glycation endproducts are associated with the development of atherosclerosis as well as diabetic microangiopathy in patients with type 2 diabetes. *Acta Diabetol* 37:87–92, 2000
11. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguci Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R: Immunohistochemical localization of advanced glycosilation endproducts in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol* 143:1649–1656, 1993
12. Yoshida N, Okumura K, Aso Y: High serum pentosidine concentrations are associated with increased arterial stiffness and thickness in patients with type 2 diabetes. *Metabolism* 54:345–350, 2005
13. Gallou G, Ruelland A, Legras B, Maugendre D, Allannic H, Cloarec L: Plasma malondialdehyde in type 1 and type 2 diabetic patients. *Clin Chim Acta* 214:227–234, 1993
14. Zheng ZJ, Sharret AR, Chambless LE, Rosamond WD, Nieto FJ, Sheps DS, Dobs A, Evans GW, Heiss G: Associations of ankle-brachial index with clinical coronary heart disease, stroke and preclinical carotid and popliteal atherosclerosis: the Atherosclerosis Risk in Communities (ARIC) Study. *Atherosclerosis* 131:115–125, 1997
15. Leng GC, Fowkes FGR, Lee AJ, Dunbar J, Housley E, Ruckley CV: Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. *BMJ* 313:1440–1444, 1996
16. Walters DP, Gatling W, Mullee MA, Hill RD: The prevalence, detection, and epidemiological correlates of peripheral vascular disease: a comparison of diabetic and non-diabetic subjects in an English community. *Diabet Med* 9:710–715, 1992
17. Hirsch AT, Criqui MH, Treat-Jacobson D, Regensteiner JC, Creager MA, Olin JW, Krook SH, Hunninghake DB, Comerota AJ, Walsh ME, McDermott MM, Hiatt WR: Peripheral arterial disease detection, awareness, and treatment in primary care. *JAMA* 286:1317–1324, 2001
18. Prineas RJ: *The Minnesota Manual of Electrographic findings*. Crow RS, Blackburn H, Eds. Bristol, Jhon Wright Press, 1982
19. American Diabetes Association: Standards of medical care in diabetes—2006 (Position Statement). *Diabetes Care* 29 (Suppl. 1):S4–S42, 2006
20. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: follow-up report on the diagnosis of diabetes mellitus. *Diabetes Care* 26:3160–3167, 2003
21. Lapolla A, Piarulli F, Sartore G, Rossetti C, Martano L, Carraro P, De Paoli M, Fedele D: Peripheral artery disease in type 2 diabetes: the role of fibrinolysis. *Thromb Haemost* 89:91–96, 2003
22. Odetti P, Fogarthy J, Sell D, Monnier VM: Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects. *Diabetes* 41:153–159, 1992
23. Makita Z, Vlassara, H Cerami A, Bucala R: Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 267:5133–5138, 1992
24. Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A: A fluorescence-based method for measuring total plasma antioxidant capacity. *Free Radic Biol Med* 18:29–36, 1995
25. Vitamins and coenzymes, part F. In *Meth-*

ods in Enzymology, vol. 67. McCormick DB, Wright LD, Eds. New York, Academic Press, 1980
26. Conti M, Morand PC, Levillain P, Lemonnier A: Improved fluorometric determination of malonaldehyde. *Clin Chem* 37: 1273–1275, 1991
27. Huggett AST, Nixon DA: Use of glucose oxidase peroxidase and O-dianisine in the determination of blood and urine glucose. *Lancet* 2:368–370, 1957
28. Jaynes PK; Willis MC, Chon PP: Evaluation of minicolumn chromatographic procedure for the measurement of HbA1c. *Clin Biochem* 39:2162–2165, 1993
29. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC: Enzymatic determination of total serum cholesterol. *Clin Chem* 20: 470–475, 1974
30. Lipid Research Clinics Program. Lipid and lipoprotein analysis. In *Manual of Laboratory Operations*, 2nd ed. Washington, DC, U.S. Department of Health and Human Services, 1982, p. 63–77
31. Fossati P, Prencipe L: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 28:2077–2080, 1982
32. Armitage P: *Statistical Methods in Medical Research*. Oxford, Blackwell Scientific Publications, 1971
33. Xia X, Xie Z: AMADA: analysis of microarray data. *Bioinformatics* 17:569–570, 2001
34. Lipkovich I, Smith EP: BiPlot and singular value decomposition macros for Excel. *J Stat Software* 7:1–15, 2002
35. Aitchinson J, and Greenacre M: Biplots of compositional data. *Applied Statistics* 51: 375–392, 2002
36. Piarulli F, Lapolla A, Sartore G, Rossetti C, Bax G, Noale M, Minicuci N, Fiore C, Marchioro L, Manzato E, Fedele D: Autoantibodies against oxidized LDLs and atherosclerosis in type 2 diabetes. *Diabetes Care* 28:653–657, 2005
37. Riemersma RA, Wood DA, Macintyre CCA, Elton RA, Gey KF, Oliver MF: Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. *Lancet* 337:1–5, 1991
38. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willet WC: Vitamin E consumption and the risk of coronary disease in women. *N Engl J Med* 328: 1444–1449, 1993
39. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Rosner B, Willet WC: Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 328:1450–1456, 1993
40. Jones AF, Winkles JW, Jennings PE, Florkoski CM, Lunec J, Barnett AH: Serum antioxidant activity in diabetes mellitus. *Diabetes Res* 7:89–92, 1988
41. Strain J: Disturbances of micronutrient and antioxidant status in diabetes. *Proc Nutr Soc* 50:591–604, 1991
42. Lonn E, Yusuf S, Hoogwerf B, Pogue J, Yi Q, Zinman B, Bosch J, Dagenais G, Mann JF Gernstein HC: Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and MICRO-HOPE substudy. *Diabetes Care* 25:1919–1927, 2002
43. The Heart Outcomes Prevention Evaluation Study Investigators: Vitamin E supplementation and cardiovascular events in high-risk patients. *N Engl J Med* 342: 154–160, 2000
44. Sacco M, Pellegrini F, Roncaglioni MC, Avanzini F, Rognoni G, Nicolucci A: Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. *Diabetes Care* 26:3264–3272, 2003
45. Osmundson PJ, O'Fallon WM, Clements IP, Kazmier BR, Palumbo PJ: Reproducibility of noninvasive tests of peripheral occlusive arterial disease. *J Vasc Surg* 2:678–683, 1985