Advanced glycation end products are associated with arterial stiffness in type 1 diabetes

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Abstract

The aim of this study was to investigate the relationship between advanced glycation end products (AGEs) and arterial stiffness (AS) in subjects with type 1 diabetes without clinical cardiovascular events. A set of 68 patients with type 1 diabetes and 68 age- and sex-matched healthy subjects were evaluated. AGEs were assessed using serum concentrations of *N*-carboxymethyl-lysine (CML) and using skin autofluorescence. AS was assessed by aortic pulse wave velocity (aPWV), using applanation tonometry. Patients with type 1 diabetes had higher serum concentrations of CML (1.18 vs 0.96 μg/ml; $P=0.008$) and higher levels of skin autofluorescence (2.10 vs 1.70; $P<0.001$) compared with controls. These differences remained significant after adjustment for classical cardiovascular risk factors. Skin autofluorescence was positively associated with aPWV in type 1 diabetes ($r=0.370$; $P=0.003$). No association was found between CML and aPWV. Skin autofluorescence was independently and significantly associated with aPWV in subjects with type 1 diabetes ($\beta=0.380$; $P<0.001$) after adjustment for classical cardiovascular risk factors. Additional adjustments for HbA1c, disease duration, and low-grade inflammation did not change these results. In conclusion, skin accumulation of autofluorescent AGEs is associated with AS in subjects with type 1 diabetes and no previous cardiovascular events. These findings indicate that determination of tissue AGE accumulation may be a useful marker for AS in type 1 diabetes.

Key Words
- advanced glycation end products
- arterial stiffness
- arteriosclerosis
- pulse wave velocity
- skin autofluorescence
- type 1 diabetes mellitus

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Introduction

Cardiovascular disease is the major cause of mortality in type 1 diabetes (Libby *et al*. 2005). Diabetes mellitus results in an accelerated arteriosclerotic process, which is not fully explained by classical cardiovascular risk factors. As a result, the pathophysiological mechanisms underlying cardiovascular events in type 1 diabetes are not completely understood.

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Arterial stiffness (AS) is an early sign of arteriosclerosis (Cavalcante *et al*. 2011) and predicts cardiovascular events independently of classical cardiovascular risk factors in several populations (Vlachopoulos *et al*. 2010). The exact mechanisms responsible for the increase in AS are not fully understood, but are likely to reflect a complex interaction between structural and functional changes in the arterial wall (Zieman *et al*. 2005). The structural changes are characterised by an overproduction of abnormal collagen and diminished quantities of normal elastin induced by an inflammatory milieu (Zieman *et al*. 2005). In this context, we have recently shown that low-grade inflammation is independently associated with an increase in AS in type 1 diabetes (Llaurado *et al*. 2012). However, other mechanisms, such as the formation of advanced glycation end products (AGEs), are also thought to be involved in the increase in AS that occurs in diabetes (Zieman *et al*. 2005).

AGEs are the result of non-enzymatic irreversible glycation and oxidation of proteins, lipids and nucleic acids. Hyperglycaemia and oxidative stress accelerate the accumulation of AGEs (Singh *et al*. 2001), which are involved in the development of micro- and macrovascular complications in type 1 diabetes (Genuth *et al*. 2005, Nin *et al*. 2011). AGEs can be assessed by chemical characterisation: pentosidine and *N*-carboxy-methyl-lysine (CML) are some examples of well-characterised AGEs. Interestingly, certain dermal AGEs exhibit autofluorescent properties and the measurement of skin autofluorescence has been proposed as a novel marker of tissue accumulation of AGEs (Meerwaldt *et al*. 2004).

The gold standard for measuring central AS is aortic pulse wave velocity (aPWV). Higher aPWV is reflective of AS. AGEs have been associated with an increase in aPWV in healthy individuals (Semba *et al*. 2009), hypertensive individuals (McNulty *et al*. 2007), subjects with end-stage renal failure (Ueno *et al*. 2008) and patients with type 2 diabetes (Choi *et al*. 2009). In type 1 diabetes, AGEs have been associated with an increase in pulse pressure, a surrogate marker for central AS (Schram *et al*. 2005). However, whether a relationship exists between AGEs and AS measured by aPWV in type 1 diabetic patients without previous clinical cardiovascular events (coronary heart disease, cerebrovascular disease or peripheral vascular disease) is not known.

The main aim of the present study was to test the strength of the relationship between two different measurements of AGEs (skin autofluorescence and serum levels of CML) and aPWV (as a surrogate for central AS) in patients with type 1 diabetes without clinical cardiovascular events.

Subjects and methods

Subjects

A set of 68 patients with type 1 diabetes, aged 18–65 years, and 68 age- and sex-matched healthy subjects were included in our study. None of the subjects had any clinical cardiovascular disease. Also, none had any condition associated with an inflammatory response (e.g. acute or chronic infectious disease) or had received anti-inflammatory treatment in the previous 6 months. Subjects with type 1 diabetes were consecutively recruited from our outpatient clinic and all had at least 1 year of evolution.

Clinical study

The study methods have previously been described in detail elsewhere (Llaurado *et al*. 2012). Briefly, after an overnight fast, venous blood samples were taken and aliquots of plasma and serum were stored at −80 °C until processed. In women, all measurements were taken during the follicular phase of the menstrual cycle. The following information was recorded using a predefined standardised form: sex, age, diabetes duration, BMI, waist-to-hip ratio (WHR), systolic and diastolic blood pressure (SBP and DBP), and mean arterial pressure (MAP) – defined as 1/3 SBP + 2/3 DBP – physical activity (International Physical Activity Questionnaire (IPAQ) (Hallal & Victora 2004)), cigarette smoking, alcohol intake, insulin dose or any other drug treatment, and microvascular complications (assessed only in patients with type 1 diabetes as previously described (Llaurado *et al*. 2012)).

Hypertension was defined as having a blood pressure of over 140/90 (Mancia *et al*. 2007) and/or being under antihypertensive treatment. Dyslipidaemia was defined as having a total cholesterol concentration of over 5.2 mmol/l, triglycerides over 1.7 mmol/l, HDL-cholesterol of less than 1.03 mmol/l, LDL-cholesterol of over 3.4 mmol/l (NCEP 2002) and/or receiving drug treatment for dyslipidaemia.

The study protocol was approved by our hospital's Ethics Committee (Sabadell Hospital Clinical Research Ethics Committee) and conducted according to the principles of the Declaration of Helsinki. All subjects gave their written informed consent before participating in the study.

Laboratory analyses

HbA1c was determined by HPLC (Menarini Diagnostics, Firenze, Italy). Total serum cholesterol, triglycerides,

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HDL-cholesterol and LDL-cholesterol were measured using standard enzymatic methods.

AGEs can act via their receptor (RAGE) triggering multiple signalling cascades, resulting in the activation of nuclear transcription factors and the secretion of pro-inflammatory cytokines and vascular adhesion molecules (Goldin *et al.* 2006). To evaluate the relationship between the three mechanisms, the main markers of low-grade inflammation, endothelial dysfunction (focusing on those markers implicated in cellular adhesion and permeability) and AGEs were evaluated. High-sensitivity C-reactive protein (hsCRP) was determined by immunonephelometry (Siemens, Munich, Germany). ELISA was used for the assessment of interleukin 6 (IL6; R&D Systems, Abingdon, UK), soluble fractions of tumor necrosis factor α receptors 1 (sTNFαR1; Hycultbiotech, Uden, The Netherlands) and 2 (sTNFαR2; R&D Systems), soluble intercellular adhesion molecule 1 (ICAM1; Boster Biological Technology, Wuhan, China), soluble vascular cell adhesion molecule 1 (VCAM1; Boster Biological Technology) and E-selectin (Boster Biological Technology). ELISA was used also for the assessment of CML (Cylex Co. Ltd, Nagano, Japan; Komosinska-Vassev *et al.* 2012). The test was carried out according to the manufacturer's instructions. The mean minimum detectable concentration was 0.126 ng/ml. The antibodies in the CML kit are highly specific for CML-adducts, with no detectable cross reactivity to non-CML proteins that may be present in human serum. The intra- and inter-assay coefficients of variation (CV) were 5.2–7.4 and 4.7–15.2% respectively for CML.

Skin autofluorescence

Skin autofluorescence was assessed using an AGE Reader (DiagnOptics BV, Groningen, The Netherlands). Three measurements per patient were taken at room temperature on the forearm, in three different positions at around 5–10 cm below the elbow fold, with patients in a sitting position. Briefly, the AGE Reader illuminates approximately 1 cm^2 of skin surface (guarded against surrounding light), with an excitation light source of 300–420 nm. The light from the skin was measured using a spectrometer (model PC-1000 fiber optic spectrometer, Ocean Optics, Dunedin, FL, USA) in the range of 300–600 nm, using 200 μm glass fibre (Farnell, Leeds, UK). Autofluorescence is calculated as the average light intensity per nanometer in the range between 420 and 600 nm, divided by the average light intensity per nanometer in the range between 300 and 420 nm (autofluorescence), multiplied by 100 and expressed as arbitrary units (AU). This technique has been previously validated for both type 1 and 2 diabetes (Meerwaldt *et al.* 2004). The CV for the three-repeated measurement was 4.2%.

Assessment of AS

Measurement of aPWV Subjects rested in supine position and measurements were taken in accordance with the recommendations of the recent consensus on AS (Laurent *et al.* 2006). The method has been previously described elsewhere in detail (Llaurado *et al.* 2012). Briefly, aPWV was determined by sequential applanation tonometry (Millar tonometer: SPC-301; Millar Instruments, Houston, TX, USA) at the carotid and femoral arteries, gated to a three-lead ECG, was recorded using the SphygmoCor device (SphygmoCor; AtCor, Sydney, Australia). Those aPWV not fulfilling the automatic quality controls specified by the SphygmoCor software were rejected. The mean of two aPWV measurements was taken for each subject for all calculations. The CV for the two repeated measurements was 3.11%. Data were available for all participants included in the study.

Statistical analyses

Data are presented as percentages, mean (S.D.) for normally distributed variables, and median (interquartile range) for non-normally distributed variables. All data were tested for normality using the Kolmogorov–Smirnov test. Differences between patients with type 1 diabetes and control patients were analysed using the χ^2-test for comparisons of proportions and unpaired *t*-tests or Mann–Whitney *U* tests for comparisons of quantitative variables as needed. Although patients were recruited to be age- and sex-matched, we performed the analyses using an independent *t*-test. We chose this strategy because it was statistically more conservative (although it is less likely to detect differences between groups). We assessed the potential relationships between all variables through univariate Spearman correlations. To assess the relationship between AGEs and AS, linear regression models were used to adjust for potential confounders, using aPWV as the dependent variable. Independent variables for linear regression analyses were selected based on univariate correlation analyses and those variables known or likely to be associated with AGEs or AS. To improve skewness and kurtosis, and to improve linearity, non-normally distributed variables were log-transformed. Collinearity tests (tolerance and the reciprocal of tolerance: the variance inflation factor) were performed first to avoid

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overlap between introduced variables (threshold for the tolerance of VIF >10). Because we measured inflammatory-related serum proteins and endothelial dysfunction markers only once, the association (if any) with AGEs and AS would tend to be underestimated. To address this issue, a *Z*-Score was calculated for each marker as follows: (value for the individual−mean value for the study population)/S.D. Subsequently, the low-grade inflammation general score was calculated as (*Z*-score for hsCRP+*Z*-score for IL6+*Z*-score for sTNFαR1+*Z*-score for sTNFαR2)/4 and an endothelial dysfunction general score was calculated as (*Z*-score for ICAM1+*Z*-score for VCAM1+*Z*-score for E-selectin)/3. The IBM SPSS Statistics package (v. 19 for Mac; SPSS, Inc., IBM Company) was used for all calculations. All *P* values were two-sided and a *P* value of <0.05 was considered statistically significant.

Table 1 Clinical characteristics of study population

	Healthy subjects (*n*=68)	**Type 1 diabetes** (*n*=68)	***P***
Age (years)	35.4 (10.2)	35.3 (10.1)	0.945[a]
Gender (male) (*n*, %)	34 (50)	34 (50)	1.00[c]
Current smokers (*n*, %)	16 (23.5)	24 (35.3)	0.252[c]
Alcohol intake	1.43 (0.00 to 5.36)	1.43 (0.00 to 5.71)	0.886[b]
Physical activity (METS, min/week)	1386.0 (784.5 to 2079.0)	1416.0 (713.3 to 2367.0)	0.791[b]
Family history			
CHD (*n*, %)	6 (8.8)	3 (4.4)	0.493[c]
T2DM (*n*, %)	12 (17.6)	16 (23.5)	0.396[c]
T1DM (*n*, %)	1 (1.5)	5 (7.4)	0.208[c]
Hypertension (*n*, %)	3 (4.4)	17 (25.0)	0.001[c]
Dyslipidaemia (*n*, %)	34 (50)	32 (47.1)	0.732[c]
Diabetes duration (years)	–	13.0 (7.3 to 19.0)	–
Microvascular complications (*n*, %)	–	16 (23.5)	–
Retinopathy (n, %)	–	10 (14.7)	–
None (*n*, %)	–	58 (85.3)	–
Non-proliferative (*n*, %)	–	6 (8.8)	–
Proliferative (*n*, %)	–	4 (5.9)	–
Nephropathy (*n*, %)	–	9 (13.2)	–
Peripheral polyneuropathy (*n*, %)	–	0 (0)	–
BMI (kg/m^2)	24.0 (3.1)	25.7 (3.6)	0.003[a]
Waist (cm)	83.7 (11.3)	85.3 (12.0)	0.429[a]
WHR	0.85 (0.1)	0.86 (0.1)	0.465[a]
Systolic blood pressure (mmHg)	120.6 (10.4)	125.0 (12.1)	0.025[a]
Diastolic blood pressure (mmHg)	70.8 (8.4)	72.9 (8.3)	0.154[a]
Mean arterial pressure (mmHg)	87.4 (8.6)	90.3 (8.7)	0.059[a]
Fasting plasma glucose (mmol/l)	4.67 (0.53)	9.15 (3.66)	<0.001[a]
Total cholesterol (mmol/l)	5.16 (1.33)	4.80 (0.87)	0.070[a]
Triglycerides (mmol/l)	0.80 (0.62 to 1.17)	0.78 (0.61 to 0.97)	0.422[b]
HDL-cholesterol (mmol/l)	1.50 (1.19 to 1.87)	1.70 (1.21 to 1.90)	0.369[b]
LDL-cholesterol (mmol/l)	2.81 (2.25 to 3.58)	2.52 (2.14 to 0.11)	0.028[b]
HbA1c (%)	5.3 (5.2 to 5.5)	7.5 (6.8 to 8.7)	<0.001[b]
Urinary ACR (mg/mmol)	0.39 (0.28 to 0.57)	0.36 (0.24 to 0.69)	0.716[b]
aPWV (m/s)	6.1 (5.5 to 6.7)	6.8 (6.0 to 7.9)	<0.001[b]
hsCRP (mg/l)	0.8 (0.4 to 1.5)	1.3 (0.5 to 2.8)	0.038[b]
IL6 (pg/ml)	0.3 (0.2 to 0.5)	0.6 (0.3 to 1.1)	<0.001[b]
sTNFαR1 (pg/ml)	1535.1 (1190.0 to 2845.6)	2592.3 (1560.8 to 3021.3)	0.014[b]
sTNFαR2 (pg/ml)	2201.8 (1880.2 to 2561.5)	2568.9 (2153.9 to 3024.0)	0.001[b]
Low-grade inflammation score	−0.35 (−0.63 to 0.16)	0.12 (−0.20 to 0.55)	<0.001[b]
ICAM1 (ng/ml)	55.1 (48.3 to 62.7)	58.1 (51.8 to 69.0)	0.063[b]
VCAM1 (ng/ml)	715.8 (541.2 to 954.7)	782.6 (628.6 to 1090.2)	0.128[b]
E-Selectin (ng/ml)	73.6 (50.9 to 99.1)	104.6 (68.5 to 184.9)	<0.001[b]
Endothelial dysfunction score	−0.28 (−0.58 to 0.02)	0.14 (−0.27 to 0.58)	<0.001[b]
CML (μg/ml)	0.96 (0.77 to 1.23)	1.2 (0.9 to 1.4)	0.008[b]
Skin AF (AU)	1.7 (1.6 to 2.1)	2.1 (1.8 to 2.3)	<0.001[b]

Data are given as mean (S.D.) (differences between type 1 diabetes and control patients were analysed using unpaired *t*-tests (normal distribution)[a], median (interquartile range) (differences between type 1 diabetes and control patients were analysed using Mann-Whitney U tests (data not-normally distributed))[b], or percentages (differences between patients with type 1 diabetes and control patients were analysed using the χ^2-test)[c]. CHD, coronary heart disease; T2DM, type 2 diabetes; T1DM, type 1 diabetes; WHR, waist-to-hip ratio; ACR, urinary albumin/creatinine ratio.

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Results

The main clinical and analytical characteristics of the studied population are given in Table 1. Regarding pharmacological medications, eight subjects were being treated with antihypertensive drugs (seven with type 1 diabetes), 15 with statins (14 patients) and six with antiplatelet drugs (all patients). Subjects with type 1 diabetes, compared with their non-diabetic counterparts, were more hypertensive and had higher values of BMI, fasting plasma glucose, and HbA1c and higher concentrations of inflammatory-related serum proteins. In addition, subjects with type 1 diabetes presented lower concentrations of LDL-cholesterol, probably due to the great number of patients treated with statins, or differences in dietary habits.

Association between circulating levels of CML and skin autofluorescence with clinical and analytical variables:

Subjects with type 1 diabetes had higher serum concentrations of CML (1.18 μg/ml (0.94–1.40) vs 0.96 μg/ml (0.77–1.23); $P=0.008$) and higher levels of skin autofluorescence (2.10 (1.80–2.30) vs 1.70 (1.60–2.10); $P<0.001$), when compared with their respective controls (Table 1). These differences remained significant after adjustment for classical cardiovascular risk factors (age, gender, physical activity, smoking, hypertension, dyslipidaemia and BMI) using multiple linear regression analysis ($\beta=0.280$; $P=0.023$ and $\beta=0.341$; $P<0.001$ for CML and skin autofluorescence respectively).

Tables 2 and 3 show univariate correlations between CML and skin autofluorescence with cardiovascular risk factors in the whole population: healthy subjects and patients with type 1 diabetes respectively. In the whole population, CML was associated with the presence of dyslipidaemia, type 1 diabetes, fasting plasma glucose and HbA1c (Table 2). Skin autofluorescence was associated with age, smoking status, the presence of hypertension and dyslipidaemia, BMI, type 1 diabetes, fasting plasma glucose and HbA1c (Table 3). In healthy subjects, CML was correlated only with the presence of dyslipidaemia (Table 2), while skin autofluorescence was positively correlated with age, total and LDL-cholesterol, and triglycerides (Table 3). In type 1 diabetes, skin autofluorescence was positively associated with age, BMI and diabetes duration ($r=0.291$; $P=0.021$; Table 3). However, no association was observed between serum concentrations of CML and cardiovascular risk factors or glycaemic control (Table 2) in these patients. No relationship was found between serum concentrations of CML and skin autofluorescence ($r=0,054$; $P=0.586$ for the whole population, $r=0.053$; $P=0.736$ for the healthy subjects and $r=0.114$; $P=0.374$ for subjects with type 1 diabetes).

Relationship between aPWV and AGE activity

Patients with type 1 diabetes had higher aPWV compared with their respective controls (6.8 (6.0–7.9) vs 6.1 (5.5–6.7); $P<0.001$). Skin autofluorescence was positively associated with aPWV only in type 1 diabetes

Table 2 Spearman correlation coefficients for the association between CML and classical cardiovascular risk factors. Data are given as Spearman correlation coefficients (*P* value)

	All	Healthy subjects	Type 1 diabetes
Age (years)	−0.092 (0.288)	−0.019 (0.875)	−0.178 (0.146)
Gender (female)	0.109 (0.206)	0.135 (0.273)	0.073 (0.552)
Diabetes mellitus (N/Y)	0.227 (0.008)	–	–
Smoking (N/Y)	0.025 (0.770)	0.174 (0.155)	−0.178 (0.147)
Physical activity (METS, min/week)	−0.111 (0.202)	−0.085 (0.492)	−0.194 (0.118)
Hypertension (N/Y)	−0.147 (0.087)	−0.066 (0.595)	−0.207 (0.116)
Dyslipidaemia	0.221 (0.010)	0.255 (0.036)	0.208 (0.089)
BMI (kg/m^2)	−0.008 (0.922)	−0.145 (0.237)	−0.033 (0.791)
Waist (cm)	−0.107 (0.216)	−0.209 (0.088)	−0.047 (0.701)
WHR	−0.063 (0.466)	−0.022 (0.859)	−0.141 (0.251)
SBP (mmHg)	−0.085 (0.326)	0.093 (0.452)	−0.189 (0.153)
DBP (mmHg)	−0.121 (0.160)	−0.049 (0.693)	−0.169 (0.202)
MAP (mmHg)	−0.124 (0.150)	−0.006 (0.985)	−0.227 (0.084)
Total cholesterol (mmol/l)	0.095 (0.270)	0.120 (0.331)	0.121 (0.327)
Triglycerides (mmol/l)	0.052 (0.547)	0.031 (0.800)	0.066 (0.593)
HDL-cholesterol (mmol/l)	0.044 (0.609)	0.032 (0.798)	0.025 (0.838)
LDL-cholesterol (mmol/l)	0.055 (0.524)	0.089 (0.470)	0.087 (0.483)
FPG (mmol/l)	0.189 (0.027)	0.042 (0.735)	0.041 (0.739)
HbA1c (%)	0.279 (0.001)	0.152 (0.215)	0.151 (0.219)

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FPG, fasting plasma glucose.

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Table 3 Spearman correlation coefficients for the association between skin autofluorescence and classical cardiovascular risk factors. Data are given as Spearman correlation coefficients (*P* value)

	All	Healthy subjects	Type 1 diabetes
Age (years)	0.444 (<0.001)	0.499 (0.001)	0.494 (<0.001)
Gender (female)	0.178 (0.067)	0.199 (0.201)	0.221 (0.082)
Diabetes mellitus (N/Y)	0.390 (<0.001)	–	–
Smoking (N/Y)	0.362 (<0.001)	0.340 (0.026)	0.241 (0.057)
Physical activity (METS-min/week)	−0.160 (0.104)	−0.124 (0.428)	−0.230 (0.074)
Hypertension (N/Y)	0.203 (0.721)	0.120 (0.445)	0.098 (0.445)
Dyslipidaemia	0.203 (0.037)	0.247 (0.111)	0.184 (0.149)
BMI (kg/m^2)	0.274 (0.005)	0.170 (0.275)	0.258 (0.041)
Waist (cm)	0.222 (0.022)	0.095 (0.545)	0.292 (0.020)
WHR	0.116 (0.235)	0.034 (0.828)	0.142 (0.266)
SBP (mmHg)	0.108 (0.269)	0.041 (0.796)	0.035 (0.784)
DBP (mmHg)	0.123 (0.209)	0.041 (0.792)	0.014 (0.911)
MAP (mmHg)	0.138 (0.159)	0.077 (0.255)	0.046 (0.721)
Total cholesterol (mmol/l)	0.175 (0.072)	0.467 (0.002)	0.126 (0.324)
Triglycerides (mmol/l)	0.107 (0.273)	0.343 (0.024)	−0.057 (0.656)
HDL-cholesterol (mmol/l)	0.094 (0.337)	0.034 (0.830)	0.162 (0.204)
LDL-cholesterol (mmol/l)	0.151 (0.123)	0.492 (0.001)	0.097 (0.449)
FPG (mmol/l)	0.305 (0.001)	0.019 (0.905)	0.049 (0.704)
HbA1c (%)	0.407 (<0.001)	0.133 (0.396)	0.193 (0.129)

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FPG, fasting plasma glucose.

patients ($r=0.370$; $P=0.003$) and not for the control group ($r=0.186$; $P=0.233$). No association between CML and aPWV was found (type 1 diabetes: $r=-0.034$; $P=0.781$ and healthy subjects: $r=-0.139$; p=0.259).

In order to strengthen the identification of the observed associations, we constructed a multiple linear regression analysis, considering aPWV as the dependent variable and adjusting for classical cardiovascular risk factors. We found that skin autofluorescence was the strongest factor associated with aPWV in type 1 diabetes ($\beta=0.380$; $P<0.001$; Table 4). Therefore, skin autofluorescence contributed to 14% of aPWV variance. Additional adjustments for HbA1c, disease duration, low-grade inflammation and endothelial dysfunction did not change these results.

Discussion

Non-invasive skin intrinsic fluorescence using the fluorescent properties of certain collagen AGEs is gaining acceptance as a novel marker of accumulation of AGEs. In this work, we show for the first time, to our knowledge, that skin autofluorescence in patients with type 1 diabetes (a high cardiovascular risk population) is one of the main factors associated with the increase of AS, determined by an accurate index such as aPWV, before development of cardiovascular disease.

AGEs are involved in the aetiology of micro- and macrovascular complications in diabetes mellitus (Wautier *et al.* 2003, Genuth *et al.* 2005, Nin *et al.* 2011). Additionally, skin autofluorescence is associated with the presence of cardiovascular disease in both type 1 and type 2 diabetes (Meerwaldt *et al.* 2007, Lutgers *et al.* 2009). Strikingly, serum levels of AGEs were predictive of cardiovascular events and all-cause mortality in a type 1 diabetes cohort (Nin *et al.* 2011). Our results are in agreement with these findings, and indicate that increased accumulation of tissue AGEs could be involved even in early stages of arteriosclerotic disease. Interestingly, skin

Table 4 Association between skin autofluorescence and aPWV in subjects with type 1 diabetes. Variables adjusted into this model were: age, gender, smoking, physical activity, hypertension (N/Y), dyslipidaemia (N/Y), BMI, mean arterial blood pressure, total cholesterol, log triglycerides, logHDL-cholesterol and skin autofluorescence. Only significant variables are shown in the table

log aPWV ($R=0.737$; $R^2=0.543$)	*B*	S.D.	β	95% CI	*P*	R^2 change
BMI	0.007	0.002	0.338	0.003–0.011	0.001	0.117
MAP	0.003	0.001	0.361	0.001–0.004	0.001	0.027
Total cholesterol	0.001	0.000	0.287	0.000–0.001	0.006	0.036
Skin autofluorescence	0.370	0.097	0.380	0.175–0.565	<0.001	0.042

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autofluorescence remained associated with AS in type 1 diabetes after adjustment for classical cardiovascular risk factors, glycaemic control, disease duration, low-grade inflammation and endothelial dysfunction. Notably, this association was specific for type 1 diabetic patients because it was not detected in healthy subjects. Although there is general agreement about age as an important determinant of both AS and AGEs, in the type 1 diabetic population we failed to find this. Probably the narrow range of age of patients included in this study hinders the detection of evidence of this association in young subjects. Interestingly, this observation reinforces the importance of obtaining a powerful and sensitive marker of arterial derangement before clinical events appear. In this sense, we are tempted to propose quantification of skin AGEs as a simple and useful tool to detect high-cardiovascular-risk subjects in early stages of type 1 diabetes. Along these lines, Conway *et al.* (2012) recently described an association between skin autofluorescence and coronary artery calcification, another measure of pre-clinical arteriosclerosis. In addition, Januszewski *et al.* (2012) showed that skin autofluorescence was correlated with the measures of systemic AS assessed as pulse wave analysis, supporting our observations.

We are aware of the descriptive results of our study, and the difficulty in inferring mechanistic hypotheses in these patients. Indeed, there are two primary mechanisms by which the accumulation of AGEs can contribute to the development of AS (Zieman & Kass 2004). On the one hand, AGEs can cause cross linking of collagen and elastin fibrils, which may lead to the loss of collagen elasticity and a subsequent reduction in arterial distensibility properties, resulting in increased AS (Sell & Monnier 2012). On the other hand, RAGE triggers multiple signalling cascades, resulting in the activation of nuclear transcription factors and the secretion of pro-inflammatory cytokines and vascular adhesion molecules (Goldin *et al.* 2006). In contrast to skin autofluorescence, CML, a non-crosslinking AGE and a ligand of RAGE, was not associated with aPWV in our study. Therefore, the lack of association between CML and AS, and the fact that the association between AS and skin autofluorescence was independent of low-grade inflammation and endothelial dysfunction markers lead us to propose the hypothesis that the effect of AGEs on AS may be mainly induced by cross linking rather than activation of the AGE–RAGE axis. However, the data in the literature are confusing on this point. Thus, some AGE-crosslink breakers, such as ALT-711, have been shown to decrease AS (Kass *et al.* 2001), while other studies have shown an association between non-crosslinking AGEs, such as CML, and AS (Schram *et al.* 2005). Thus, new studies are required before definite conclusions concerning the specific role of the different types of AGEs in the vascular pathology of type 1 diabetes can be reached. Differences in the degree of protein modification by accumulation of AGEs, rather than on the specific AGEs *per se*, may be an alternative explanation for these discrepancies.

We are aware that the major limitation of our study is its cross-sectional design, which makes it impossible to determine the predictive value of skin autofluorescence, and undoubtedly additional prospective studies are needed. As we have stated, although patients were recruited together with age- and sex-matched controls, we performed the analyses using an independent *t*-test. We chose this strategy because unpaired analyses were statistically more conservative. Thus, the lack of significant differences between the groups might be either because there was no difference between them, or because there was a difference but the statistical analysis chosen did not detect it. There are some technical limitations in determination of skin autofluorescence. Not all AGEs exhibit fluorescent properties and other tissue components that fluoresce in the same range might be confounders. However, the method has been validated against specific AGE levels in skin biopsies in healthy subjects as well as in patients with diabetes (Meerwaldt *et al.* 2004). In addition, fluorescence is a group reactivity, and does not allow for the collection of quantitative information on the individual compounds responsible for it. In addition, we measured only one type of circulating AGE and it was measured only once. This might have underestimated the association between AGEs and AS. In relation to glycemic control, other studies have found that skin AF correlated with mean Hb1Ac values (5 and 10 years previously) but not with the latest value (Genevieve *et al.* 2013). In our study, we only analysed the latest HbA1c value; probably this accounts for the lack of association between glycemic control and skin AF. Nevertheless, we believe that these criticisms do not reduce the relevance of the association reported in our study.

In conclusion, circulating AGEs, measured as CML plasma levels, and skin accumulation of AGEs, are increased in patients with type 1 diabetes without clinical cardiovascular disease. Skin autofluorescence is associated with AS independently of classical cardiovascular risk factors, glycaemic control, disease duration and low-grade inflammation. These findings indicate that the

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determination of tissue AGE accumulation may be a useful marker for AS in type 1 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declarations about their contributions: G L researched data, wrote the manuscript, contributed to the discussion and reviewed/edited the manuscript; V C-M researched data; C V contributed to the discussion and reviewed/edited the manuscript; R S contributed to the discussion and reviewed/edited the manuscript; P G researched data; A C researched data; J V contributed to the discussion and reviewed/edited the manuscript; J-M G-C wrote the manuscript, contributed to the discussion and reviewed/edited the manuscript.

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