

Carotid artery intima media thickness associates with skin autofluorescence in non-diabetic subjects without clinically manifest cardiovascular disease

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ABSTRACT

Background Skin autofluorescence (skin AF) is determined in part by accumulation of advanced glycation end products. Increased skin AF was shown previously to predict cardiovascular events independently of conventional risk factors. We determined the association of carotid artery intima media thickness (IMT), a marker of subclinical cardiovascular disease, with skin AF in subjects without diabetes or clinically manifest cardiovascular disease.

Methods In a cross-sectional observational study, IMT, skin AF, lipids and apolipoproteins, C-reactive protein (CRP), insulin resistance and paraoxonase-1 activity were measured in 59 non-smoking, non-obese subjects without diabetes mellitus and cardiovascular disease (32 women; 12 subjects with metabolic syndrome (MetS)).

Results In univariate analyses, skin AF was correlated with IMT ($r = 0.265$, $P = 0.042$), but not significantly with clinical factors, (apo)lipoproteins, CRP, insulin resistance and paraoxonase-1. In multiple linear regression analyses, IMT was determined independently by age ($\beta = 0.549$, $P < 0.001$), apo B ($\beta = 0.236$, $P = 0.022$) and skin AF ($\beta = 0.216$, $P = 0.035$). IMT was also associated with skin AF ($\beta = 0.213$, $P = 0.046$) in a model which included the presence of MetS.

Conclusions IMT is positively related to skin AF, independently of clinical factors, (apo)lipoproteins and MetS, suggesting that skin AF represents a determinant of subclinical atherosclerosis. Increased skin AF may reflect early abnormalities in processes involved in atherosclerosis development.

Keywords Intima media thickness, lipoproteins, metabolic syndrome, paraoxonase-1, skin autofluorescence.

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Introduction

Advanced glycation endproducts (AGEs) comprise a group of irreversibly modified proteins, lipids and nucleic acids that result from non-enzymatic glycation and oxidative processes [1,2]. Hyperglycaemia and oxidative stress accelerate the accumulation of AGEs throughout the body. AGE levels are increased in atherosclerotic lesions of subjects with and without diabetes mellitus, which favour the possibility that AGEs may play a role in the pathogenesis of atherosclerosis [3–5]. Non-invasive measurement of skin autofluorescence (skin AF) has been proposed as a marker of accumulation of AGEs in the skin [6]. This method takes advantage of the ability of skin tissue to emit specific light wavelengths in response to excitation light [5]. We have reported that skin AF is increased in type 1 and

type 2 diabetes mellitus in conjunction with microvascular and macrovascular complications [7,8], in stable coronary artery disease [9], as well as during the acute phase of myocardial infarction [10]. Moreover, it has been demonstrated that skin AF predicts increased incidence of cardiovascular disease (CVD) in type 2 diabetic patients [11], whereas increased skin AF is also a determinant of recurrent CVD in post-myocardial infarction patients [10].

The association of increased skin AF with incident and recurrent cardiovascular events is independent of conventional cardiovascular risk factors [8,10]. This raises the possibility that increased tissue AGE accumulation reflects abnormalities in early stages of atherosclerosis development. So far, information

regarding the possible relationship of skin AF with intima media thickness (IMT), an established marker of subclinical atherosclerosis [12], is limited. Skin AF was found to be correlated positively with carotid artery IMT in patients with systemic lupus erythematosus [13], and with femoral artery IMT in pregnant women with and without previous pre-eclampsia [14]. A positive relationship of skin AF with carotid IMT was also found in a small group of young adults [15], but not in another group of healthy subjects [13].

This study was initiated to determine to which extent carotid artery IMT is related to skin AF in non-smoking, non-obese subjects without diabetes mellitus and clinically manifest cardiovascular disease.

Materials and methods

Reporting of the study conforms to STROBE and the broader Equator guidelines [16].

This cross-sectional study was performed in a university hospital setting. The medical ethics committee of the University Medical Centre Groningen, The Netherlands approved the study, and all participants provided written informed consent.

The participants (aged > 18 years) were Caucasian, and were recruited by advertisement in local newspapers during a 12 month period. Previously diagnosed diabetes mellitus, cardiovascular disease, renal insufficiency (elevated serum creatinine and/or proteinuria), treatment for hypertension, thyroid disorders, liver disease, as well as current pregnancy were exclusion criteria. Physical examination did not reveal pulmonary or cardiac abnormalities. Of the 67 potentially eligible participants, subjects with BMI > 30 kg m⁻², smokers and subjects who used > 3 alcoholic drinks per day were also excluded. This was performed to reduce potential confounding caused by possible effects of obesity, smoking and excessive alcohol consumption on subclinical atherosclerosis and on the quantity and quality of plasma lipoproteins. As a result 59 subjects participated in this study. All subjects were studied after an overnight fast.

BMI was calculated as weight divided by height squared (in kg m⁻²). Waist circumference was measured between the 10th rib and the iliac crest. Homeostasis model assessment (HOMA_{IR}) was used as a measure of insulin sensitivity, and was calculated with the formula: fasting plasma insulin × glucose/22.5 [17]. Metabolic syndrome (MetS) was defined according to the revised NCEP-ATP III criteria [18]. Three or more of the following criteria were required for categorization of subjects with MetS: waist circumference > 102 cm for men and > 88 cm for women; hypertension (blood pressure ≥ 130/85 mmHg); fasting plasma triglycerides ≥ 1.7 mmol L⁻¹; HDL cholesterol < 1.0 mmol L⁻¹ for men and < 1.3 mmol L⁻¹ for women; fasting glucose ≥ 5.6 mmol L⁻¹.

Carotid IMT measurement

IMT of the carotid arteries was determined using ultrasonography in the supine position. High-resolution B-mode ultrasound images were obtained (ACUSON 128 XP, Mountain View, CA, USA) with a 7.5 MHz linear array transducer. Three arterial wall segments in each carotid artery were imaged from a fixed lateral transducer angle at the far wall. The segments scanned were the segment 1 cm proximal to the carotid dilatation (common carotid artery), the segment between the carotid dilatation and carotid flow divider (carotid bulb) and a 1 cm segment distal to the flow divider (internal carotid artery). The scans were recorded on S-VHS tape and analysed off-line by an image analyst who was unaware of subjects' characteristics. B-mode image analyses were digitized with a frame grabber (DT286 I; Data Translation Inc.; Marlboro, MA). The image analysis software used an algorithm that was developed by Selzer *et al.* [19]. The mean IMT of 6 carotid artery segments was calculated and used for analysis. At a mean IMT of 0.80 mm, inter-sonographer variability was 0.05 mm, with image analyst variability < 0.03 mm, corresponding to a total variation coefficient between 6.3% and 7.3%.

Skin autofluorescence

Skin autofluorescence was assessed with the Excitation-Emission Matrix Scanner (EEMS), an adapted set-up of the AGE Reader that was used in several previous studies of our group. The EEMS set-up assesses skin AF similar to the AGE Reader but has the additional potential to discriminate between AF spectra obtained at various excitation wavelengths. A previous report showed that AF values obtained with the EEMS are slightly lower than AF values measured with the AGE Reader which can be explained by some differences between both set-ups [13].

The EEMS technique and set-up have been described in detail elsewhere [20]. Briefly, approximately 4 cm² of the skin of the ventral site of the lower arm is illuminated by a computer driven system with a 75W Xenon lamp and a 0.2 m f/4 monochromator (PTI, NJ, USA). A series of measurements is thereby obtained for each subject, varying the peak excitation wavelength from 360 to 405 nm with 5 nm interval steps. To derive the mean skin AF from the excitation-emission matrices, firstly the AF values for each selected peak excitation wavelength were calculated by dividing the mean emitted intensity per nm in the range between 420 and 600 nm by the mean reflected excitation intensity per nm between 300 and 420 nm for a given excitation wavelength and expressed in arbitrary units (AU). Subsequently, mean skin AF was determined by calculating for each excitation step the contribution in excitation light intensity corresponding to that of a conventional AGE Reader lamp intensity spectrum with a maximum wavelength at 370 nm.

The AF value was calculated off-line by automated analysis and is observer-independent. As skin colour can also influence the AF measurement, the skin AF value was corrected for reflection of the skin when reflection was below 12%.

Laboratory analyses

Venous blood samples for measurement of lipids, lipoproteins, apolipoprotein (apo)A-I, apoB, insulin, high sensitive C-reactive protein (CRP) were collected into EDTA-containing tubes (1.5 mg mL⁻¹). Serum was obtained for measurement of paraoxonase-1 (PON-1) activity. Samples were prepared by centrifugation at 1400 g for 15 min at 4 °C. Glucose was measured shortly after blood collection. Samples for other assays were kept frozen at -80 °C until analysis.

Plasma cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11 876 023 and 11 875 540 respectively, Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi). Non-HDL cholesterol was calculated as the difference between plasma total cholesterol and HDL cholesterol. Apo A-I and apo B were determined by immunoturbidimetry (Roche/Cobas Integra Tina-quant cat nos 03 032 566 and 03 032 574 respectively, Roche Diagnostics Inc., Mannheim, Germany). Serum PON-1 activity was measured as its arylesterase activity, corresponding to the rate of hydrolysis of phenyl acetate into phenol [21,22]. Arylesterase was expressed in kU per litre of serum, with 1 U being equivalent to 1 µmol of phenyl acetate hydrolyzed per min. Glucose was analysed with an APEC glucose analyser (APEC Inc., Danvers, MA, USA). Plasma insulin was determined with a microparticle enzyme immunoassay (AxSYM Insulin assay; Abbott Laboratories, Abbott Park, IL, USA). Glycated haemoglobin (HbA1c) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, the Netherlands; reference range 4.6–6.1%). High-sensitive-CRP (CRP) was assayed by nephelometry with a lower limit of 0.175 mg L⁻¹ (BNII N; Dade Behring, Marburg, Germany).

Statistical analysis

Data are provided as mean ± SD or as median (interquartile range). Univariate relationships were calculated using linear regression analysis. Variables that were correlated with IMT at a *P*-value of < 0.05 or < 0.10 were included in the multivariate analysis. Multiple linear regression analysis followed by subsequent backward elimination was carried out to disclose the statistically independent associations of IMT with skin AF and other variables. Backward elimination was accomplished with an *F*-value based *P*-value for entry into the model of 0.049 and of 0.05 for removal. Because of skewed distribution, logarithmically transformed values for CRP, triglycerides, insulin and

HOMA_{ir} were used. Two-sided *P*-values < 0.05 were considered to be statistically significant.

Results

Fifty-nine predominantly middle-aged subjects (mean age 55 ± 10 years) participated in the study. Clinical characteristics, mean carotid IMT, skin AF, plasma glucose, insulin, HOMA_{ir}, HbA1c, CRP, plasma lipids and apos, as well as serum PON-1 activity are shown in Table 1. IMT was higher in men than in women (0.84 ± 0.16 vs. 0.76 ± 0.13, *P* = 0.029), whereas skin AF did not differ between genders (1.59 ± 0.40 vs. 1.54 ± 0.42, *P* = 0.62). Twelve subjects (20.3%) fulfilled the revised NCEP ATP III criteria for MetS. The same 12 individuals would have been classified with MetS when the original NCEP ATP III 2001 criteria were applied. Subjects with and without MetS did not show significant differences in IMT (0.85 ± 0.16 mm vs.

Table 1 Clinical characteristics, carotid intima media thickness (IMT), skin autofluorescence (skin AF), HbA1c, insulin resistance, C-reactive protein (CRP), plasma lipids and apolipoproteins (apos), and paraoxonase-1 (PON-1) activity in 59 subjects

Age (years)	55 ± 10
Sex (Male/Female)	27/32
Carotid IMT (mm)	0.80 ± 0.15
Skin autofluorescence (AU)	1.57 ± 0.41
Body Mass Index (kg m ⁻²)	24.9 ± 2.50
Waist (cm)	85 ± 11
Systolic blood pressure (mmHg)	130 ± 20
Diastolic blood pressure (mmHg)	82 ± 12
HbA1c (%)	5.3 ± 0.4
Fasting glucose (mmol L ⁻¹)	5.7 ± 0.6
Insulin (mU L ⁻¹)	5.7 (4.5–7.7)
HOMA _{ir} (mU × mmol/(L ² × 22.5))	1.41 (1.03–2.04)
CRP (mg L ⁻¹)	1.00 (0.48–1.96)
Cholesterol (mmol L ⁻¹)	5.88 ± 0.92
Non-HDL cholesterol (mmol L ⁻¹)	4.31 ± 1.00
HDL cholesterol (mmol L ⁻¹)	1.57 ± 0.40
Triglycerides (mmol L ⁻¹)	1.22 (0.88–1.80)
Apo B (g L ⁻¹)	0.97 ± 0.24
Apo A-I (g L ⁻¹)	1.47 ± 0.22
PON-1 activity (kU L ⁻¹)	130 ± 39

Data in mean ± SD and in median (25th to 75th percentile). HOMA_{ir}, homeostasis model assessment.

0.78 ± 0.13 mm, $P = 0.15$) or in skin AF (1.52 ± 0.41 AU vs. 1.58 ± 0.41 AU, $P = 0.64$).

The univariate correlations of IMT and skin AF with clinical variables, HbA1c, insulin sensitivity, CRP, plasma lipids, apos and serum PON-1 activity are shown in Table 2. IMT correlated significantly and positively with age, waist circumference, systolic blood pressure and apoB, whereas skin AF was not significantly correlated with any of the variables listed in Table 2. In univariate analysis, IMT correlated positively with skin AF ($r = 0.265$, $P = 0.042$).

Multiple linear regression analysis was performed to establish independent associations of IMT with skin AF. In a multivariate model which included those variables with which IMT was correlated in univariate analysis at a P -value < 0.05 (Table 2), IMT was found to be independently related to skin AF, age and apoB (Table 3, model 1; R-square = 0.455). Results were similar when this analysis was repeated with inclusion of variables that were univariately related to IMT at a P -value < 0.10 (data not shown). In a second model, it was determined whether the relationship of IMT with skin AF was modified by

Table 2 Univariate relationships of carotid artery intima media thickness (IMT) and skin autofluorescence (skin AF) with clinical variables, HbA1c, insulin resistance, C-reactive protein (CRP), plasma lipids and apolipoproteins (apos), and paraoxonase-1 (PON-1) activity in 59 subjects

	Carotid IMT	Skin AF
Age	0.596****	0.092
BMI	0.228*	0.085
Waist	0.395***	0.186
Systolic blood pressure	0.268**	0.091
Diastolic blood pressure	-0.047	-0.067
HbA1c	-0.172	-0.00
Fasting glucose	0.151	0.152
Ln HOMA _{ir}	0.14	0.095
Ln CRP	0.028	-0.047
Plasma cholesterol	0.194	-0.027
Non-HDL cholesterol	0.229*	-0.034
HDL cholesterol	-0.129	0.026
Ln triglycerides	0.137	-0.140
Apo B	0.299**	-0.00
Apo A-I	-0.016	0.01
PON-1 activity	-0.118	-0.090

Pearson's correlation coefficients are shown. BMI: body mass index; HOMA_{ir}: homeostasis model assessment. * $P < 0.10$, ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

Table 3 Multiple linear regression models providing relationships of carotid artery intima media thickness (IMT) with clinical variables (age, sex, systolic blood pressure, metabolic syndrome (MetS), apolipoprotein (apo) B and skin autofluorescence (AF) in 59 subjects

	Model 1 β	P -value	Model 2 β	P -value
Age	0.488	< 0.001	0.520	< 0.001
Age	0.549*	< 0.001	0.576*	< 0.001
Sex (M vs. F)	0.037	0.76	0.117	0.291
Skin AF	0.191	0.070	0.216	0.044
	0.216*	0.035	0.213*	0.046
Waist	0.092	0.46		
Systolic blood pressure	0.116	0.275		
Apo B	0.204	0.063		
	0.236*	0.022		
MetS (yes/no)			0.095	0.38

Model 1 includes age, sex, skin AF, waist, systolic blood pressure and apolipoprotein (apo) B. Model 2 includes age, sex, skin AF and the presence of MetS. Independent statistical determinants of IMT at a P -value < 0.05, as assessed by subsequent backward are marked (*). β , standardized regression coefficient.

the presence of MetS. This analysis demonstrated a positive association of IMT with skin AF and age, independently of the presence of MetS (Table 3, model 2; R-square = 0.400). This association of IMT with skin AF remained significant ($P < 0.05$ for all analyses) when the five individual MetS components were each separately included in the model (data not shown).

Discussion

This cross-sectional study demonstrates that carotid artery IMT is positively correlated with skin AF in non-obese, non-smoking subjects without diabetes mellitus and cardiovascular disease. This positive relationship was independent of waist circumference, systolic blood pressure and apolipoprotein B, variables to which IMT was significantly correlated in univariate analysis. Furthermore, the association of skin AF with IMT remained significant when the presence of the metabolic syndrome was taken into account. Our findings are in agreement with the hypothesis that increased skin AF reflects at least in part processes that contribute to early stages of atherosclerosis development.

We applied several criteria for subject exclusion, including prevalent diabetes, clinically manifest cardiovascular disease, renal insufficiency, obesity and smoking, to reduce possible confounding with respect to possible effects on IMT and/or skin AF. Nevertheless, 20% of the participants in our study ful-

filled the criteria for metabolic syndrome. In comparison, in a population-based study carried out in the same region of the Netherlands, 20–22% of subjects, aged 50 to 59 years, were classified with metabolic syndrome [23]. Thus, it is unlikely that there is important selection with respect to the cardiovascular risk factor profile between this study participants and the general population. Although the association of metabolic syndrome with IMT did not reach significance in this report, IMT was positively correlated with waist circumference, systolic blood pressure and apolipoprotein B in univariate analysis, in keeping with a contribution of adiposity, systemic haemodynamics and atherogenic lipoproteins to IMT thickening [24–26].

In the presently evaluated predominantly middle-aged subjects, no significant relation of skin AF was found with age, blood pressure, insulin resistance, CRP and plasma (apo)lipoproteins. In the previous reports, skin AF was found to be correlated positively with age [7,10]. CRP was associated with AF in subjects with recent myocardial infarction, but not in subjects with stable CVD [9,10]. Positive relationships of skin AF with BMI and HbA1c, and negative relation of AF with HDL cholesterol have been observed in a diabetic population [7]. The reasons for absence of such correlations in the currently studied cohort are uncertain, but could be attributable to the criteria that we applied for participation or to insufficient power.

Of considerable interest, it was reported recently that the HbA1c level, a readily available measure of circulating protein glycation, predicts future coronary heart disease and stroke, even after controlling for fasting plasma glucose [27]. That study was performed in a community-based population of 11 092 non-diabetic adults. In our study, we found no correlation of HbA1c with IMT or skin AF as markers for cardiovascular disease. This difference can largely be explained by the considerable difference in sample size. Furthermore, the mean BMI in subjects with the highest category HbA1c: 6.0–6.5% and > 6.5%, were respectively 30.0 and 32.5 kg m⁻², whereas in our study subjects with BMI > 30 kg m⁻² were excluded.

Paraoxonase-1 (PON-1) is an atheroprotective enzyme which promotes degradation of lipid peroxides and prevents pro-inflammatory responses to oxidized lipids [22,28,29]. As anti-oxidative processes could affect lipid peroxide accumulation, we determined the association of skin AF with serum PON-1 activity, measured as its arylesterase activity; this activity is strongly related to PON-1 mass levels [21,22]. The absence of a correlation with skin AF suggests that circulating PON-1 levels do not modify to an important extent accumulation of tissue AGEs in healthy subjects.

Some methodological issues need to be addressed. Firstly, the set-up to assess skin AF that we currently used (EEMS) is slightly different to AF measurements using the AGE-Reader. Although the physical principles are similar, the absolute AF values obtained by these set-ups cannot be directly compared.

That this study showed no relation between age and skin AF is unlikely attributable to the difference in AF assessment, because a previous study did show a relation between AF (EEMS) and age [13]. Secondly, not all glycation products exhibit fluorescence after illumination. However, skin AF has shown to be related to tissue levels of certain fluorescent (pentosidine) as well as non-fluorescent (carboxymethyllysine and carboxyethyllysine) AGEs [6]. The latter two products can also be formed during lipoxidation reactions. It is not exactly identified which fluorescent advanced lipoxidation products contribute to skin AF. The observation that skin AF is related to serum levels of the soluble isoform of AGEs (sRAGE) implicates involvement of activation of oxidative and inflammatory pathways [9,30]. These findings provide a pathophysiological rationale to determine the contribution of skin AF to progression of atherosclerosis development.

In conclusion, the positive relationship of skin AF with intima media thickness in non-diabetic subjects without cardiovascular disease, as observed in this study, suggests that increased skin AF could reflect early abnormalities in processes involved in atherosclerosis development.

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Conflict of interest

R. Graaff and A. J. Smit are founders and stockholders of Diagnostics Technologies B.V., The Netherlands, manufacturer of the AGE Reader.

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