



Skin autofluorescence is elevated in patients with stable coronary artery disease and is associated with serum levels of neopterin and the soluble receptor for advanced glycation end products

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Abstract

Aims: To investigate whether skin autofluorescence (AF), a non-invasive marker for advanced glycation end products (AGEs), is elevated in stable coronary artery disease (sCAD) and to investigate its relationship with serum levels of the soluble receptor for AGEs (sRAGE), neopterin and C-reactive protein (CRP).

Methods and results: Skin AF and serum levels of sRAGE, neopterin and CRP were assessed in 63 sCAD patients (mean age: 64.7 ± 10.5 years), comprising 78% males, 19% subjects with diabetes, and 22% current smokers and in 33 (mean age: 63.4 ± 10.0 years) healthy non-diabetic and non-smoking age and gender matched controls. Skin AF was significantly increased in sCAD compared with controls, irrespective of diabetes, current smoking and renal function. Levels of sRAGE (standardized β : 0.43 (explaining 17% of variance in skin AF); $P < 0.001$), neopterin (β : 0.36 (11%); $P = 0.003$) and glucose (β : 0.29 (8%); $P = 0.0011$) as well as current smoking (β : 0.26 (6%); $P = 0.024$) were independently associated with skin AF (R^2 0.42), whereas the association of gender, former smoking, body mass index, CRP, lipids, creatinine clearance and pulse pressure with skin AF was not significant in this model.

Conclusion: These data demonstrate that skin AF is elevated in sCAD and is related to sRAGE and neopterin, making it an easily applicable tool to improve our understanding of inflammatory and oxidative stress in cardiovascular disease.

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Keywords: Autofluorescence; Coronary artery disease; Glycosylation end products; Advanced; Advanced glycosylation end products receptor; Neopterin; Oxidative stress; Inflammation

1. Introduction

It has recently been established that in addition to classical mechanisms, oxidative modification of carbohydrates and lipids enhances the formation of reactive carbonyl species, which are capable of transforming proteins to irreversible highly stable compounds, generally referred to as advanced glycation end products (AGEs). Although, classically associ-

ated with diabetes and renal failure [1], these compounds also appear to play a pivotal role in acute and chronic atherosclerotic disease, by means of structural protein changes in the vascular wall, but also by activation of cellular receptors, such as the receptor for AGEs (RAGE) leading to activation of several oxidative and inflammatory pathways [2–4].

We have recently introduced the AGE-Reader, a device that rapidly and non-invasively assesses accumulation of AGEs, making use of their fluorescence characteristics [5]. It measures autofluorescence emitted from the human skin (skin AF) and has been validated to specific AGEs measured

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in human skin biopsy samples in several patient groups and healthy controls [6,7]. Skin AF is elevated in patients with diabetes or renal failure, especially in those with atherosclerotic disease [8]. We hypothesized that skin AF is elevated in patients with stable coronary artery disease (sCAD) and aimed to investigate the association of skin AF with serum levels of the soluble isoform of RAGE (sRAGE), and with two well validated inflammatory markers, neopterin (as an index of monocyte activation) and C-reactive protein (CRP; as a general marker of systemic inflammation).

2. Methods

2.1. Subjects

This cross-sectional study was performed between January 2005 and November 2005, and included 63 patients with sCAD admitted for elective coronary angiography (CAG) and 33 age and gender matched healthy controls. sCAD was defined as typical chest pain, or a history of an acute coronary syndrome (ACS), or vascular intervention, combined with the presence of at least one coronary artery with mild stenosis (>30% luminal narrowing) on CAG. Healthy controls had normal femoral and carotid arteries on echo duplex. The following exclusion criteria applied for all subjects: recent ACS (<3 months), known renal disease or serum creatinine >150 $\mu\text{mol/l}$, current inflammatory or malignant disease and skin photo type V or VI (i.e. coloured skin) or skin reflectance <12%. Clinical data were obtained by chart review and questionnaires, and all measurements were performed at admission before CAG for sCAD subjects. Diabetes mellitus was defined by criteria from the American Diabetes Association. Hypertension and dyslipidemia were not categorized because of a frequent use of antihypertensives and lipid lowering drugs in sCAD patients for treatment of angina. Smoking was defined as smoking at least five cigarettes daily. Creatinine clearance (CrCl) was estimated using the Cockcroft–Gault formula. This study complied with the Declaration of Helsinki and was approved by the local ethics committee; all subjects gave written informed consent.

2.2. Assessment of skin AF

Skin AF was assessed on the ventral site of the lower arm with a prototype of the current AGE-Reader (DiagnOptics BV, Groningen, The Netherlands). This method has been extensively described elsewhere [5]. In short, the AGE-Reader consists of a 29 cm \times 13 cm \times 9 cm (length \times width \times height) box, containing an excitation light source (4 W blacklight, Philips) emitting light with wavelengths of 300–420 nm (peak \sim 360 nm). Light is transmitted through a 4 cm² large window on the upper side of the box, directly illuminating the skin. Only light reflected and emitted from the skin is measured with an integrated spectrometer (Avantes Inc., Eerbeek, The Netherlands) in a 300–600 nm range, using

a 50 μm glass fiber (Avantes Inc.). Additionally, dark and white reference measurements are performed before every measurement to correct for dark current background light and to calculate reflectance, respectively. All actions are performed automatically. Each AF measurement is composed of the average of 50 (individual) scans, each of approximately 200 ms, depending on skin reflectance. The entire AF measurement takes approximately 30 s to be performed. To correct for differences in light absorption, skin AF is calculated by dividing the mean value of the emitted light intensity per nm between 420 and 600 nm by the mean value of the excitation light intensity per nm between 300 and 420 nm, expressed as arbitrary units (AU) [5]. The intra-individual percent Altman error is 5.0% on a single day and 5.9% for seasonal changes [6].

2.3. Laboratory assessments

Venous blood was collected in the morning after an overnight fast. Lipid concentrations (total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol and triglycerides), glucose and creatinine were measured by routine techniques from fresh plasma.

2.4. Serum biomarkers

For measurement of biomarkers, blood was centrifuged at 2700 rpm for 10 min and serum was stored in 2 ml cryotubes at -20°C until batch laboratory assessment. Serum levels of neopterin (Brahms, Henningsdorf, Germany) and sRAGE (R&D Systems, Minneapolis, USA) were measured using commercially available enzyme linked immunosorbent (ELISA) techniques, according to manufacturer's instructions. CRP was determined in a high sensitive sandwich ELISA using unconjugated and horseradish peroxidase conjugated polyclonal antibodies (DakoCytomation, Glostrup, Denmark). Details have been described elsewhere [9]. All methods had an inter- and intra-assay variation of less than 5%.

2.5. Statistical analysis

We determined that a sample size of 50 patients and 30 matched controls would have 85% power to detect a difference of at least 15% at $\alpha=0.05$. Normal distribution of variables was tested with the Kolmogorov–Smirnov test. Descriptive statistics are presented as mean values \pm S.D., as median (inter-quartile range) for skewed variables or as percentages. For comparison between groups, continuous variables were analysed by Student's *t*-test. In case of categorical variables, the chi-square test or Fisher's exact test was used. To test whether differences in skin AF could be explained by differences in potential confounders, one-way analysis of covariance (ANCOVA) was performed and *F* statistic and estimation of effect size (eta-squared, η^2) were calculated. For univariate correlations, skewed variables were log transformed for a better linear fit, and Pearson correla-

tion coefficient (r) was given for continuous variables. Where appropriate partial correlations are given, corrected for confounders. Stepwise, forward selection was used to construct a multivariate model with skin AF as the dependent variable and age, gender, current and former smoking, sRAGE, neopterin, glucose, BMI, CRP, lipids, CrCl and pulse pressure as independent variables. Variables with P -values >0.10 were removed from the model. A two-sided P -value <0.05 was considered statistically significant. All statistical analyses were carried out with the Statistical Package for Social Science (SPSS, version 12.0.2, 24 March 2005).

3. Results

3.1. Subject characteristics

Patients and matched controls did not differ in age and gender distribution, with the majority being male. Skin reflectance was significantly higher in patients compared with controls. More details on subject characteristics are outlined in Table 1. Patients had an extensive medical history of vascular disease: 27% had a previous acute coronary syndrome (ACS), 35% had a previous percutaneous coronary intervention and 27% had a coronary artery bypass graft. Additionally, 5% had a previous stroke and 13% peripheral artery disease. The percentage of patients and controls that fell in the three categories of CRP levels (i.e. <1 , 1 – 3 and >3 mg/l) as suggested by the American Heart Association did not differ significantly between groups (controls: 16, 44, 41%; patients: 19, 22, 59%; $P=0.091$).

3.2. Differences in skin AF and serum biomarkers between groups

Skin AF was significantly higher in patients compared with controls (Table 2; Fig. 1). Correcting for differences in skin reflectance did not, significantly, influence the difference in skin AF between groups (skin reflectance: $F=0.18$, $\eta^2=0.002$, $P=0.67$; sCAD: $F=8.4$, $\eta^2=0.084$, $P=0.005$;

Table 1
Clinical characteristics of study groups

	sCAD ($n=63$)	Controls ($n=33$)	P -values
Age (years)	64.7 ± 10.5	63.4 ± 10.0	0.57
Men	49 (78%)	23 (72%)	0.53
Skin reflectance (%)	20 ± 5	16 ± 4	<0.001
Smoking behaviour			
Current	14 (22%)	0	0.002
Former	43 (68%)	23 (72%)	0.72
Diabetes mellitus	12 (19%)	0	0.007
BMI (kg/m^2)	27.7 ± 4.3	25.5 ± 3.4	0.012
CrCl ($\text{ml}/\text{min}/1.73 \text{ m}^2$)	82.9 ± 28.3	83.5 ± 23.1	0.92
Blood pressure			
Systolic (mmHg)	144	136	0.057
Diastolic (mmHg)	77	83	0.015
Pulse pressure (mmHg)	68	53	<0.001
Statin use	52 (84%)	1 (3%)	<0.001
Antihypertensive use	62 (98%)	3 (9%)	<0.001
β -Blocking agents	48 (77%)	0	<0.001
Diuretics	7 (11%)	1 (3%)	0.19
ACE-inhibitors	19 (30%)	2 (6%)	0.008
ARBs	8 (13%)	0	0.048
Calcium antagonists	33 (52%)	0	<0.001
Aspirin use	46 (73%)	0	<0.001

Values are mean \pm S.D. or number subject and percentage; sCAD: stable coronary artery disease; BMI: body mass index; CrCl: creatinine clearance (Cockcroft–Gault formula); ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker. Differences between groups were tested with Student's t -test, chi-square test or Fisher's exact test where appropriate.

corrected skin AF 2.21). Correction for other potential confounders, including diabetes ($F=5.3$, $\eta^2=0.054$, $P=0.024$), current smoking ($F=0.79$, $\eta^2=0.009$, $P=0.38$), body mass index ($F=1.9$, $\eta^2=0.02$, $P=0.18$) or renal function ($F=1.1$, $\eta^2=0.012$, $P=0.3$), decreased mean skin AF in the patient group to 2.15 ± 0.52 , 2.21 ± 0.60 , $2.09 \pm 0.41 \times 10^{-2}$ and 2.22 ± 0.59 AU, respectively, but skin AF remained significantly higher compared with controls (all $P<0.05$). Patients did not have significantly higher levels of CRP, sRAGE and neopterin compared with controls. Since neopterin correlated with CrCl ($r=-0.37$; $P=0.001$), neopterin values were

Table 2
Biomarkers

	sCAD ($n=63$)	Controls ($n=33$)	P -values
CRP (mg/l)	4.1 (1.6–7.8)	2.9 (1.5–5.5)	0.20
Neopterin (nmol/l)	7.5 ± 2.0	7.1 ± 1.7	0.33
sRAGE (pg/ml)	1373 ± 653	1299 ± 419	0.51
Glucose (mmol/l)	5.3 (4.7–6.1)	4.8 (4.6–5.3)	0.024
Cholesterol (mmol/l)	4.4 ± 0.8	5.7 ± 0.8	<0.001
Triglycerides (mmol/l)	1.8 (1.4–2.2)	1.1 (0.8–1.7)	<0.001
HDL-cholesterol (mmol/l)	1.2 ± 0.2	1.6 ± 0.4	<0.001
LDL-cholesterol (mmol/l)	2.3 ± 0.6	3.6 ± 0.6	<0.001
Cholesterol/HDL-cholesterol	3.8 ± 0.8	3.8 ± 1.0	0.74
Creatinine ($\mu\text{mol}/\text{l}$)	95 ± 15	89 ± 10	0.045

Values are mean \pm S.D., medians (inter-quartile range); sCAD: stable coronary artery disease; AF: autofluorescence; CRP: C-reactive protein; sRAGE: soluble receptor for advanced glycation end products; HDL: high density lipoprotein and LDL: low density lipoprotein.

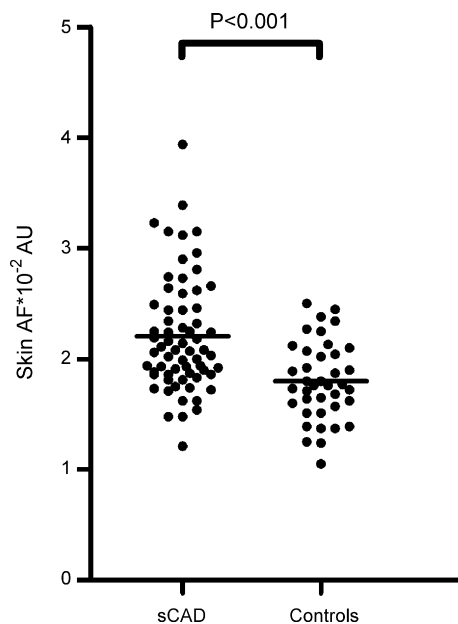


Fig. 1. Comparison of skin autofluorescence (AF) levels between patients with stable coronary artery disease (sCAD) and healthy age and gender matched controls. Horizontal line represents the mean. AU: arbitrary units.

divided by CrCl for comparison between groups. These corrected values were higher in sCAD compared with matched controls (0.12 ± 0.06 versus 0.10 ± 0.03 ; $P = 0.008$).

3.3. Factors potentially related to skin AF

In patients and controls analysed as a whole, skin AF correlated with age ($r = 0.24$; $P = 0.0017$), body mass index ($r = 0.21$; $P = 0.0038$), and pulse pressure ($r = 0.25$; $P = 0.0016$), but also with serum sRAGE ($r = 0.39$; $P < 0.0001$), neopterin ($r = 0.29$; $P = 0.005$) and glucose ($r = 0.26$; $P = 0.0017$). Skin AF tended to be higher in current smokers (2.31 ± 0.54 versus 2.06 ± 0.48 ; $P = 0.079$), and correlated inversely with total cholesterol ($r = -0.24$; $P = 0.018$) and LDL-cholesterol ($r = 0.28$; $P = 0.007$). However, this relation diminished after correction for statin use. There was no significant correlation of skin AF with CRP, creatinine and CrCl. When analysing the patient group, only sRAGE (Fig. 2A) and neopterin (Fig. 2B), were significantly associated with skin AF.

Table 3 demonstrates that when analysing patients and controls as a whole or patients only, stepwise linear regression

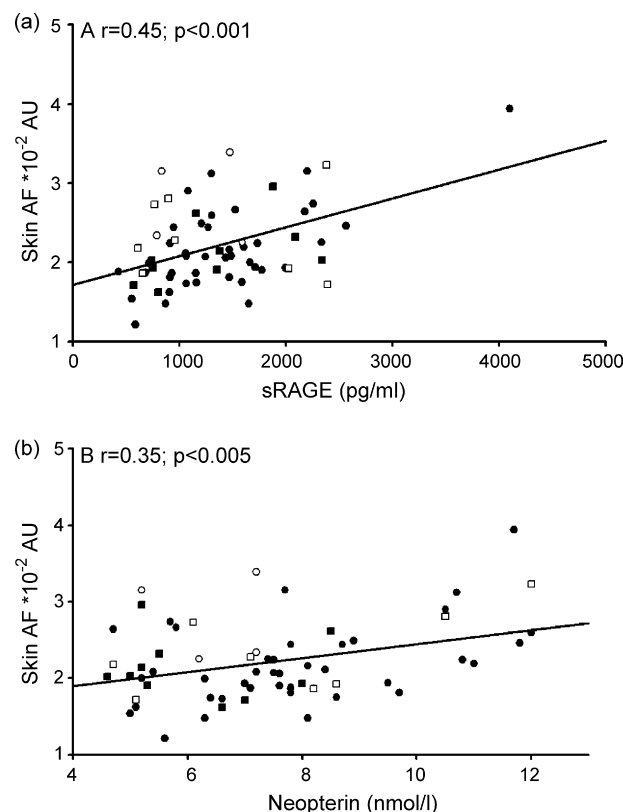


Fig. 2. Scatter plots of the association of skin autofluorescence (AF) with the soluble receptor for advanced glycation end products (sRAGE) (A) and neopterin (B) in patients with stable coronary artery disease (sCAD). Black circle reflects non-smoking patients without diabetes, open circle reflects smoking patient with diabetes, black square reflects smoking patients without diabetes and open square reflects non-smoking patients with diabetes.

identified an independent association of skin AF with only sRAGE, neopterin, glucose and current smoking (R^2 was 0.38 in patients and controls as a whole and 0.42 in patients only). Age, gender, former smoking, BMI, CRP, lipids, CrCl and pulse pressure were not significantly associated with skin AF in these multivariable regression models.

4. Discussion

In this study, we demonstrate that skin AF is elevated in patients with sCAD compared with age and gender matched controls. Even after correction for diabetes, smok-

Table 3
Stepwise multiple regression analysis of variables independently associated with skin AF

	All subjects			Patients only		
	β	95% CI	P-values	β	95% CI	P-values
sRAGE	0.40	0.22–0.59	<0.001	0.43	0.21–0.66	<0.001
Neopterin	0.32	0.14–0.51	0.001	0.36	0.12–0.59	0.003
Glucose (log)	0.25	0.06–0.43	0.009	0.29	0.07–0.50	0.011
Current smoking	0.28	0.09–0.46	0.005	0.26	0.04–0.49	0.024

sCAD: stable coronary artery disease; sRAGE: soluble receptor for advanced glycation end products; CI: confidence interval.

ing, or impaired renal function this difference persisted. Furthermore, in patients with sCAD, skin AF was independently and positively associated with serum levels of the soluble receptor for AGEs (sRAGE) and neopterin. Since sRAGE and neopterin are both considered to be involved in enhanced cellular oxidative and/or glycaemic stress these data provide insight in the role oxidative, inflammatory and glycaemic stress in the development of cardiovascular disease.

In previous reports, we have already demonstrated that skin AF is strongly related to skin accumulation of AGEs, as evidenced by a high correlation with specific AGEs measured from skin biopsy homogenates [6]. Since these measured AGEs included either exclusively carbohydrate or ascorbic acid derived AGEs (pentosidine), but also mainly lipid derived AGEs (carboxymethyllysine and carboxyethyllysine), we concluded that skin AF may be a non-invasive marker for both glycaemic and oxidative stress [10]. This was also supported by the observation that skin AF was inversely related to plasma vitamin C levels, a strong antioxidant, in subjects with renal failure [11]. Furthermore, subjects with diabetes and particularly those with neuropathy or micro- and macro-vascular complications and subjects with renal failure had significantly higher levels of skin AF [6,8,12,13]. Most importantly, skin AF predicted CAD related mortality in patients with renal failure independently established risk factors [14]. The data from the present study are in agreement with our previous observations; however, prospective data are needed to investigate the clinical usefulness of skin AF in predicting future events in patients with stable CAD.

Through interaction with their major cellular receptor, RAGE, AGEs may prime proinflammatory mechanisms in monocytes and endothelial cells, thereby amplifying proinflammatory mechanisms in atherosclerotic plaque formation [15]. Engagement of RAGE results in intracellular signaling, which leads to sustained activation of the proinflammatory transcription factor NF- κ B resulting in enhanced expression of inflammatory mediators. Sustained activation of this cascade also leads to up regulation of the transmembrane receptor [16]. It has been suggested that proteolysis of full-length RAGE may be a mechanism for the increase in the level of the soluble isoform in the serum. However, more research is warranted to elucidate the mechanisms of sRAGE formation [17]. Recently, the key role of RAGE in the generation of oxidative stress and subsequent cellular damage was pointed out in an animal model of ischemia/reperfusion injury after myocardial infarction. It was demonstrated that lipidperoxidation derived AGEs are generated by ischemia/reperfusion and subsequently activate RAGE, augmenting vascular and inflammatory cell activation. Animals lacking cellular expression of RAGE were more likely to be protected from RAGE mediated damage [4]. In clinical studies, Falcone et al. reported lower sRAGE levels in subjects with sCAD [18], and Koyama et al. found sRAGE to be inversely correlated with intima media thickness [19]. It was hypothesized that sRAGE functions as a circulating decoy receptor and binding to its ligands decreases

its free form, resulting in lower serum levels measured with the ELISA kit. In our study, sCAD patients had sRAGE levels similar to those of healthy controls. The marked difference between our sRAGE results and those of the considerably larger study group of Falcone et al. may well be due to the differences in study groups: while they studied only non-diabetic males without lipid lowering drugs, in our group females (22%) and diabetes patients (19%) were also represented while the large majority (84%) used statins. Recently, sRAGE levels have been found to be elevated in other diseases associated with oxidative stress, such as renal failure [17] and acute lung injury [20]. Moreover, our results are in line with the a study by Yamagishi et al. who found serum levels of sRAGE to be positively associated with circulating AGEs in a non-diabetic general population [21]. Because our study was not primarily designed to investigate the issue of sRAGE differences between sCAD patients and controls, it is difficult to draw definite conclusions from our data. Furthermore, it is important to note that several ligands other than AGEs also bind to RAGE [16].

In chronic kidney disease, enhanced expression of RAGE on circulating monocytes is strongly correlated with plasma levels of tumor necrosis factor alpha, neopterin and CRP [22]. Additionally, it has been reported that plasma levels of neopterin are associated with pentosidine levels in patients with chronic kidney disease [23]. These data are in agreement with our findings that skin AF, as a validated marker for AGEs, is strongly associated with the soluble isoform of RAGE, reflecting RAGE expression, as well as with neopterin, reflecting macrophage activation.

Surprisingly, CRP levels were not significantly higher in sCAD patients compared with matched controls and CRP was not related to skin AF. A logical explanation might be that most of our patients were receiving intensive drug treatment with statins, antihypertensive agents and aspirin. We have previously shown that statin treatment modulates CRP levels as well as neopterin [24]. Additionally, some antihypertensive agents and aspirin may also decrease oxidative stress and inflammation [25].

As reported by others, neopterin appeared to be higher in patients, after correction for CrCl [26]. In vitro, neopterin exhibits distinct biochemical effects, most likely via interactions with reactive oxygen or nitrogen intermediates, thereby affecting the cellular redox state [27]. In clinical studies, neopterin levels were elevated in patients with ACS [28]. Therefore, neopterin can be regarded as a marker for disease activity, and may also serve as a risk marker for future cardiovascular events in patients with sCAD [29] as well as with ACS [30].

4.1. Study limitations

Since this study had a cross-sectional design, a causative relation between skin AF and serum markers cannot be confirmed. From previous investigations we have learned that skin AF cannot be reliably measured with current equipment

in subjects with skin photo type V–VI or reflectance <12% [5]. Therefore, for this study, we excluded these skin types and research for improving the measurement is ongoing.

Since not all AGEs encompass fluorescent properties, skin AF is only representative of part of the total AGE burden. However, in our validation study, we found that skin AF also correlated strongly with non-fluorescent AGEs [6]. Additionally, two major lipid peroxidation products, 4-hydroxynonenal and malondialdehyde – after binding to free aminogroups of protein – also encompass characteristic fluorescent properties [31]. Since the AGE-Reader covers a wide excitation/emission spectrum, skin AF measured with the AGE-Reader may have a miscellaneous origin.

5. Conclusion

To the best of our knowledge, this is the first study to demonstrate that a non-invasive marker for inflammatory and oxidative stress is elevated in predominantly euglycemic subjects with sCAD. Skin AF was independently and strongly associated with serum levels of sRAGE, a marker for enhanced cellular expression of this receptor as well as with neopterin, as a marker for monocyte activation. We hypothesize that skin AF may be a non-invasive index of inflammatory and oxidative stress in patients with sCAD. Although, prospective studies are warranted to confirm this hypothesis, skin AF may provide an easily applicable tool to improve our understanding these important modulators of atherosclerotic cardiovascular diseases.

6. Competing interest

Graaff and Smit are co-inventors in a patent application concerning the autofluorescence reader and are stockholders in the company DiagnOptics BV, which develops and produces the AGE-Reader, which is based on the prototype used in the present article. The other authors have nothing to declare.

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