Novel visualization techniques towards identification of atherosclerotic patients at risk
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 13-01-2020
CHAPTER 4

Skin autofluorescence – a marker for advanced glycation end products – is positively associated with plasma levels of matrix metalloproteinase in subjects without overt atherosclerosis.


Submitted for publication
OBJECTIVES Advanced glycation end products (AGEs) have been shown to influence the in vitro production of matrix metalloproteinases (MMPs), thereby propagating atherosclerosis development. We tested the association between skin auto fluorescence (skin AF, a non-invasive technique to measure tissue AGEs), inflammation (hsCRP), sRAGE and plasma levels of MMPs as well as different stages of atherosclerotic plaque development (measured by IMT) in healthy individuals.

METHODS In 262 healthy subjects with a Framingham Risk score ranging form 0 to 15%, skin AF, mean and maximum intima media thickness (IMT) of the common carotid artery was measured. Subjects were divided in three groups; subjects without atherosclerosis (plaque score 0), intermediate atherosclerosis (plaque in 1-3 segments) and atherosclerosis in more than 4 segments (high, plaque score ≥ 4). Furthermore, plasma levels of sRAGE and hsCRP were measured. Also serum levels of MMPs (MMP-3, MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were determined.

RESULTS Skin AF correlated with MMP-3 ($r=0.26$, $P<0.0001$), MMP-9 ($r=0.31$, $P<0.0001$), TIMP-1 ($r=0.25$, $P<0.0001$) and hsCRP ($r=0.26$, $P<0.0001$) in univariate analyses. In contrast, no correlation between skin AF and sRAGE could be found. Furthermore, skin AF correlated with mean IMT ($r=0.35$, $P<0.0001$) and maximal IMT ($r=0.17$, $P=0.008$), and FRS scores were related to skin AF. Skin AF and biomarkers were significantly higher in subjects with atherosclerotic plaque (intermediate, score of 1-3), compared to subjects without atherosclerosis in the carotid arteries. MMP-9 and hsCRP were significantly lower in the intermediate plaque group compared to the highest group.

CONCLUSION Skin AF is positively associated with MMPs and TIMP-1 in plasma and with FRS. MMP-9 had the best correlation with skin AF in univariate analysis, and was significantly different in all three plaque groups. Skin AF together with plasma MMP-9 might be a good indicator for cardiovascular risk in subjects without overt atherosclerosis.
SKIN AF AND PLASMA MMPS IN SUBJECTS WITHOUT OVERT ATHEROSCLEROSIS

Introduction

Atherosclerosis is an inflammatory disease mediated by cellular mechanisms driven by oxidative stress (1). Advanced glycation end products (AGEs) are stable structures accumulating on long-lived proteins, formed by non-enzymatic glycation and oxidative reactions (2). AGEs are implicated in long term complications of diabetes mellitus and renal disease, and evidence for an important role in cardiovascular disease beyond these conditions is growing (3). By interaction with their major receptor (RAGE), AGEs have been shown in vitro to promote inflammatory mechanisms leading to atherosclerotic plaque formation and vulnerability (4). However, little clinical evidence is available to substantiate these observations.

Matrix metalloproteinases (MMPs) play an important role in virtually all stages of atherosclerosis development (5). Already in early stages of plaque formation, MMP production by inflammatory cells induces basal membrane degradation, promoting smooth muscle cell migration and plaque development. Furthermore, extensive literature has demonstrated that MMP activity, especially in the shoulders of plaques, leads to instability ensuing acute cardiovascular events including ischemic stroke and acute coronary syndromes (6). In vitro (7) and ex vivo (8,9), evidence is growing that AGE production is linked to the MMP metabolism. AGEs co-localize in intimal areas of both aortic and coronary specimens and by engagement with RAGE MMP expression on inflammatory cells is induced (10). These events contribute to plaque destabilization by inducing culprit MMP expression and may potentially play an important role in atherosclerotic plaque development and instability.

In clinical studies, measurement of AGEs in plasma is difficult. We developed and extensively validated a non-invasive technique to measure tissue AGEs by evaluating skin autofluorescence (skin AF) (11). Skin AF is indeed a strong predictor of cardiovascular (CV) mortality (12). Furthermore, earlier studies have indicated that skin AF is elevated in coronary artery disease, irrespective of diabetes mellitus (DM) or renal disease (13), correlates with carotid intima media thickness (IMT) (14), and is elevated in patients with carotid artery stenosis and peripheral artery disease (PAD) (15). Furthermore, skin AF was shown to be associated with CRP in patients with several conditions, including central obesity (16), DM (17), hemodialysis (18) and coronary artery disease (19), and correlated with the soluble isotype of RAGE (sRAGE) in coronary artery disease (19) and inversely related to HDL anti-oxidative capacity in DM (20).

Taking the above mentioned evidence into account, we believe that AGEs might play an important role in the inflammatory process leading to atherosclerosis development. The present study was initiated to test whether plasma levels of MMP-3, -9, TIMP-1 and hsCRP and sRAGE, as well as atherosclerotic plaque development (measured by IMT), are associated with tissue levels of AGEs (measured by skin AF) in subjects without cardiovascular disease (CVD), but with a wide range in Framingham Risk Scores (FRS).
Materials and Methods

Study design
This study involved 262 healthy subjects of at least 18 years of age. To obtain a wide range of FRS in subjects without a history of CVD, subjects were recruited from two cohorts, i.e. a healthy group of subjects recruited by advertisement with no more than one cardiovascular risk factor (RF) and a second healthy group of subjects that participated in an international cohort, referred to as the IMPROVE study (21) who were selected on the presence of at least three RFs (dyslipidemia, hypertension, DM, smoking, or family history of cardiovascular disease). We measured skin AF, performed carotid ultrasound examinations for assessment of intima media thickness of the carotid artery and measured plasma levels of MMP-3, -9 and TIMP-1, sRAGE, and hsCRP. All measurements were compared to skin AF. Participants had to be asymptomatic for cardiovascular diseases and free of any conditions that might limit IMT visualization. Patients with a life expectancy ≤ 3 years were excluded from participation (in accordance with IMPROVE guidelines (21)). Furthermore, subjects with an estimated glomerular filtration rate of <60 ml/min/1.73 cm² or a history of renal transplantation, recent acute coronary syndrome, cerebrovascular attack or sepsis (all within the past 3 months), and current cancer or autoimmune disease were excluded because these conditions have previously been shown to increase skin AF. Furthermore, participants with a brown or black skin type (Fitzpatrick skin type V and VI) (22) had to be excluded as skin AF could not be measured reliably due to excessive light absorption in the skin. The study was approved by the local institutional review board and all participating subjects gave written informed consent.

Risk factor definitions
The following RFs were assessed (as formulated in the original IMPROVE study protocol): smoking status, dyslipidaemia (low density lipoprotein (LDL) cholesterol >4.0 mmol/l, high density lipoprotein (HDL) cholesterol <1.2 mmol/l (female) or HDL-cholesterol <1.0 mmol/l (male), triglycerides > 4.0 mmol/l, or current lipid lowering treatment, hypertension (≥140/90 mmHg or drug treatment for hypertension), obesity (body mass index (BMI) ≥30 kg/m²), and DM (known diabetes or fasting plasma glucose >7.0 mmol/l or random plasma glucose >11.1 mmol/l). The Framingham risk score was defined as the 10 year risk on coronary heart disease (i.e. myocardial infarction or death from coronary heart disease) (23). Kidney function was estimated using the MDRD (‘modification of diet in renal disease’) formula.

Biochemical analyses
Blood was collected after overnight fasting. Venous blood samples were collected into EDTA-containing tubes (1.5 mg /mL). 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. Additional plasma samples were kept at –80°C prior to analysis at the lab. HsCRP was determined by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg, Germany). Serum levels of sRAGE (R&D Systems, Minneapolis, USA) and MMP-3 (Biosource, Nivelles, Belgium) were measured using commercially available enzyme linked immunosorbent (ELISA) techniques, according to manufacturer’s instructions. MMP-9 and TIMP-1 were determined as described before (24). All methods had an inter- and intra-assay variation of less than 5%.

**Carotid artery atherosclerosis assessment using ultrasonographic variables**

Carotid ultrasonography was performed using two closely related methods, as described earlier (21,25). Briefly, the far walls of the left and right common carotids, bifurcations, and internal carotids were visualized in anterior, lateral, and posterior projections and recorded on sVHS videotapes. All carotid measurements were averaged to calculate the mean IMT for each subject; the highest IMT value among the three carotid segments was defined as the maximal IMT. Repeatability of carotid IMT measurements was previously described (21). Presence of plaque was based on the Mannheim consensus statement (26), where a plaque is a focal structure that encroaches onto the arterial lumen at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness of ≥ 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. A total plaque score was calculated summing all segments with presence of at least one plaque, consequently ranging from 0 to 6. Subjects where divided in three groups; subjects without atherosclerosis (plaque score 0, n= 64), intermediate atherosclerosis (plaque in 1-3 segments, n= 166) and atherosclerosis in more than 4 segments (high, plaque score ≥ 4, n= 32). The sonographers were unaware of the characteristics and biomarker levels of the studied persons. The measurements were analyzed offline by an independent image analyst who was unaware of the clinical status of the patient.

**Measurement of skin autofluorescence**

Skin AF was assessed with the AGE Reader (DiagnOptics Technologies BV, Groningen, the Netherlands). This is a non-invasive desk-top device that uses the characteristic fluorescent properties of certain AGEs to estimate the level of AGEs accumulation in the skin by illuminating a skin surface of 4 cm². The method has been previously validated to skin biopsy dermal tissue homogenates (27). Technical details concerning the optical technique have been extensively described elsewhere (28). Skin AF is calculated from three consecutive measurements of the right forearm as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units (AU). Earlier, skin AF was shown to have an intra-individual Blant-Altman error of 5.0% on a single day, and 5.9% for seasonal variation (11).
Statistical analysis

Medians with interquartile range for nonparametric distributions and mean with standard deviation for Gaussian populations were provided. The association between skin AF and plasma biomarkers and IMT was calculated using the Pearson or Spearman correlation when appropriate. Also an ordinal logistic regression (univariate and multivariate) analysis using stepwise selection (inclusion criterium $P<0.01$) method to examine the relationship and significance between skin AF, MMP-3 and -9, TIMP-1 and hsCRP additional to classical risk factors associated to skin AF (age, diabetic status, smoking status and BMI) was done. All statistical analyses were performed using PASW Statistics version 22 (SPSS Inc, Chicago, Illinois, USA). All statistical tests were two-sided. A P-value of less than 0.05 was considered statistically significant.
Results

Subject characteristics
A total of 124 men and 138 women with a median age of 60.2 (19-81.5) years were included. The clinical and laboratory characteristics of the study population are given in Table 1. A significant correlation of skin AF with several characteristics was observed, including age ($r=0.36$, $P<0.0001$), obesity ($P=0.0004$), BMI ($r=0.35$, $P<0.0001$), presence of DM ($P<0.001$) and plasma glucose levels ($r=0.26$, $P<0.0001$). Skin AF was negatively correlated with HDLC levels ($r=-0.23$, $P=0.0002$).

Table 1. Baseline characteristics and risk factors for atherosclerosis

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Subjects (n=269)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men, n (%)</td>
<td>128 (48)</td>
</tr>
<tr>
<td>Age, years</td>
<td>60.2 (19-81.5)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.8 ± 5.2</td>
</tr>
<tr>
<td>Obesity, n (%)</td>
<td>75 (32)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td>41 (15)</td>
</tr>
<tr>
<td>Past, n (%)</td>
<td>163 (61)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>185 (69)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>146 ± 20</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>83 ± 10</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>175 (65)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.3 (0.5-5.6)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.3 ± 1.1</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>33 (12)</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>4.8 (2.7-13.8)</td>
</tr>
</tbody>
</table>

Medication

<table>
<thead>
<tr>
<th>Medication</th>
<th>Subjects (n=269)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin use, n (%)</td>
<td>74 (28)</td>
</tr>
<tr>
<td>ACEi/ARB use, n (%)</td>
<td>52 (19)</td>
</tr>
<tr>
<td>Other antihypertensive medication</td>
<td>150 (56)</td>
</tr>
<tr>
<td>Aspirin use</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Antidiabetic use</td>
<td>21 (8)</td>
</tr>
<tr>
<td>Insulin use</td>
<td>5 (2)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation or median (range) when appropriate. Percentages between brackets. ACEi = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker.

Skin autofluorescence (AGEs) vs plasma levels of MMPs, TIMP, hsCRP, and sRAGE
MMP-3 and -9 levels, hsCRP and TIMP-1 were positively correlated to skin AF (figure 1). No correlations were found for skin AF compared to plasma sRAGE levels.
Figure 1. Skin AF was positively correlated to plasma levels of (A) MMP-3 (B) MMP-9 (C) TIMP-1 and (D) hsCRP.

Univariate analyses showed plasma levels of MMP-3, -9, TIMP-1, and hsCRP were significantly associated with skin AF ($P<0.001$ for each). The same yields for the risk factors: BMI, DM, age, and smoking. Also MMP-3 and MMP-9 correlated to TIMP-1 ($r=0.37$, $P<0.001$ and $r=0.41$, $P<0.001$ respectively), and MMP-3 and MMP-9 correlated to each other ($r=0.19$, $P=0.003$). Multivariate linear regression analysis separately for MMP-3, MMP-9, TIMP-1 and hsCRP, together with the risk factors DM, age, BMI and smoking, showed significant association with skin AF for MMP-3 ($P<0.001$), MMP-9 ($P=0.05$) and TIMP-1 ($P=0.05$, Table 2). HsCRP was not independently associated with skin AF in this model ($P=0.544$).
Mean IMT (figure 2A) as well as maximal IMT (highest IMT value among the three carotid segments, figure 2B) correlated significantly with skin AF. All biomarkers were associated to mean IMT (P<0.001 for MMP-3, MMP-9 and TIMP-1, P=0.01 for hsCRP). Also, max IMT was correlated to MMP-9 and TIMP-1 (P=0.02 for both). No correlation could be found between max IMT and MMP-3 and hsCRP. Also the 10 year risk on myocardial infarction or death from coronary heart disease was correlated to skin AF, measured using the Framingham risk score (shown in figure 2C).

**Table 2. Regression analysis of MMPs and TIMP-1 associated with skin AF, independently of age, DM, BMI, smoking and hsCRP**

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95%CI</th>
<th>Beta</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3 (ng/mL)</td>
<td>0.007</td>
<td>0.004</td>
<td>0.009</td>
<td>0.328</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)</td>
<td>0.005</td>
<td>0.003</td>
<td>0.006</td>
<td>0.336</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>0.009</td>
<td>0.005</td>
<td>0.012</td>
<td>0.313</td>
</tr>
<tr>
<td><strong>Multivariate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3 (ng/mL)*</td>
<td>0.004</td>
<td>0.002</td>
<td>0.006</td>
<td>0.200</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)*</td>
<td>0.002</td>
<td>0.000</td>
<td>0.004</td>
<td>0.125</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)*</td>
<td>0.003</td>
<td>0.000</td>
<td>0.007</td>
<td>0.118</td>
</tr>
<tr>
<td>DM (yes/no)</td>
<td>0.002</td>
<td>0.001</td>
<td>0.004</td>
<td>0.160</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.006</td>
<td>0.004</td>
<td>0.008</td>
<td>0.355</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>&lt;0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.290</td>
</tr>
<tr>
<td>Smoking (yes/no)</td>
<td>0.001</td>
<td>0.000</td>
<td>0.003</td>
<td>0.086</td>
</tr>
<tr>
<td>hsCRP (mg/mL)</td>
<td>&lt;0.001</td>
<td>-0.002</td>
<td>0.001</td>
<td>-0.052</td>
</tr>
</tbody>
</table>

Univariate and multiple regression analysis using stepwise selection method. * MMP-3, -9 and TIMP-1 were analyzed separately, as they were closely related. B indicates the unstandardized coefficients with 95%-confidence interval (95%CI). Beta indicates standardized coefficient. BMI: body mass index, MMP-3: matrix metalloproteinase-3. MMP-3 and hsCRP were log transformed, natural logarithm of age was used.
Figure 2. Skin AF was correlated to (A) mean intima media thickness and, (B) max intima media thickness. The 10 year risk of cardiovascular disease using the Framingham Risk Score (FRS) is positively correlated to skin AF.
Figure 3A shows skin AF is significantly higher in subjects with atherosclerotic plaque (intermediate, score of 1-3), compared to subjects without atherosclerosis in the carotid arteries (score of 0, P=0.004). No differences were found between the intermediate group and the group with atherosclerosis in ≥ 4 segments. In the intermediate plaque group, MMP-3, MMP-9, TIMP-1 and hsCRP levels were significantly higher compared to subjects without atherosclerosis in the carotid arteries (score of 0, P<0.001 for all). MMP-9 and hsCRP were significantly lower in the intermediate plaque group compared to the highest group (P=0.04 and P=0.01 respectively, shown in figure 3B and C). No differences were found in MMP-3 and TIMP-1 between these groups.
Figure 3. (A) Skin AF is higher in subjects with intermediate plaque (atherosclerosis in 1-3 segments) compared to subjects without plaque (0 segments). No difference was found between the intermediate and high group (≥ 4 segments) (B) MMP-9 is higher in subjects with intermediate plaque compared to subjects without plaque (P=0.0008), and higher in the high group compared to the intermediate group (P=0.04). (C) hsCRP is higher in subjects with intermediate plaque compared to subjects without plaque (P=0.0001), and higher in the high group compared to the intermediate group (P=0.01).
Discussion

The current study demonstrates that skin AF, which is a non-invasive measurement for tissue AGEs, is positively associated with plasma levels of MMP-3, -9, TIMP-1, hsCRP, Framingham risk score and IMT in subjects without cardiovascular disease. These data suggests the importance of AGEs in atherosclerotic plaque formation and potentially plaque vulnerability, as MMPs trigger plaque rupture. MMP-3, MMP-9 and TIMP-1 were associated with skin AF independent of known confounders, including age, BMI, smoking and DM, but more importantly independent of CRP as a marker for inflammation. Now, examination of carotid arteries for intima-media thickness (IMT) is a widely used and non-invasive marker to reveal the severity of atherosclerosis and evaluation of vascular stenosis. However, this conventional diagnostic imaging method is not sufficient; as the chance for an atherosclerotic plaque to cause clinical symptoms is equal to its probability to become vulnerable and rupture. Factors that promote plaque vulnerability such as presence of lipid pools and necrotic core with an overlying thin fibrous cap, increased macrophage activity, production of MMPs and plaque progression, are observed within plaques that are modest in terms of angiographic stenosis severity within positively remodeled arterial segments (29).

Based on previous research, MMP-9 seems to play the most important role in atherosclerosis. In a study by Loftus et al., patients undergoing carotid endarterectomy were investigated on the character, level, and expression of MMPs in carotid plaques. This was correlated to their clinical status. It was found that MMP-9 concentration was significantly higher in symptomatic patients compared to asymptomatic patients. No difference in the levels of MMP-1, -2, or -3 was found between symptomatic and asymptomatic patients (30). Furthermore, another study reported segments at or near the bifurcation and segments with intraplaque hemorrhage contained higher MMP-9 levels and activity compared to segments distant from the bifurcation. TIMPs were highly abundant in fibrotic and necrotic segments. Histologically stable plaques contained lesser amounts of MMPs, which were predominantly MMP-2 (31). sRAGE was not correlated to skin AF. A unified formula that takes AGEs, sRAGE, and esRAGE (endogenous secretary RAGE) into consideration might be a better biomarker than sRAGE alone for all AGE-RAGE-associated diseases, as these biomarkers react dependently (32).

To the best of our knowledge, this is the first study to demonstrate the association between non-invasively assessed tissue AGEs and plasma levels of MMP-3, 9 and TIMP-1 in vivo. However, plasma levels of AGEs have been shown to correlate with plasma levels of MMP-9 in patients with myocardial infarction before (33). Also a study by Ishibashi et al., showed MMP-9 expression was correlated to AGE accumulation histologically in autopsy specimens from the aortae and coronary arteries (34), and Cipollone et al. showed atherosclerotic plaques from diabetic patients had higer RAGE and MMP-9 expression found by immunohistochemistry (35). These and our results are in line with previous
studies demonstrating a relation between skin AF with inflammation, in autoimmune diseases which are strongly inflammation driven. In systemic sclerosis, skin AF correlated with CRP (36) and in rheumatoid arthritis skin AF correlated with vWF and sVCAM-1 (37). More importantly, skin AF correlated with CRP in diseases accompanied by low-grade inflammation, including in central obesity (16), Type 1 DM (17), and in hemodialysis (HD) patients (18). Furthermore, skin AF was inversely related to HDL anti-oxidative capacity in type 2 DM (20).

These data add more evidence to earlier published research, demonstrating a strong relation between skin AF and atherosclerosis. Skin AF is increased in and a strong predictor of mortality in DM and end stage renal disease (38) but it is also elevated in patients with ST-elevated myocardial infarction (39). In the latter, skin AF was also prospectively associated with major adverse cardiac events, demonstrating a potentially etiological role of the AGE burden as measured by skin AF in atherosclerosis progression. This study shows a relation between skin AF and MMPs, which play an important role in the development of a vulnerable atherosclerotic plaque.

Matrix metalloproteinases play an important role in virtually all stages of atherosclerosis development, and are related to AGEs. AGEs upregulate NF-κB, promoting inflammatory cascades, leading to CRP and MMP production. In vitro AGEs were shown to promote the expression of MMPs on monocytes (40) and human glomerular mesangial cells (41). This leads to pathological tissue damage, such as retinal damage in diabetic retinopathy (42), a process that could be reversed by an MMP inhibitor (43).

In conclusion, plasma levels of MMP-3, -9, TIMP-1 and hsCRP were correlated to skin AF. The biomarkers named above and skin AF were both associated with plaque score and IMT in subjects with a variable CVD risk profile. MMP-9 and hsCRP were significantly different in all three plaque groups. Based on previous research, MMP-9 seems to play the most important role in atherosclerosis. This might indicate that skin AF together with plasma MMP-9 is a good clinical biomarker for subclinical atherosclerosis in risk groups such as DM or arthritis patients.

Acknowledgements
We would like to thank the IMPROVE study team for their assistance in this study.

Conflict of interest statement
Dr. Smit is founder and stockholder of DiagnOptics Technologies B.V., The Netherlands, which is the manufacturer of the AGE Reader. All other authors have no conflicts of interest to declare.
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Part 2
IMAGING OF ATHEROSCLEROSIS