Skin Autofluorescence Associates With Vascular Calcification in Chronic Kidney Disease

Angela Yee-Moon Wang, Chun-Kwok Wong, Yat-Yin Yau, Sharon Wong, Iris Hiu-Shuen Chan, Christopher Wai-Kei Lam

Objective—This study aims to evaluate the relationship between tissue advanced glycation end products, as reflected by skin autofluorescence, and vascular calcification in chronic kidney disease.

Approach and Results—Three hundred patients with stage 3 to 5 chronic kidney disease underwent multislice computed tomography to estimate total coronary artery calcium score (CACS) and had tissue advanced glycation end product assessed using a skin autofluorescence reader. Intact parathyroid hormone (P<0.001) displaced estimated glomerular filtration rate as third most significant factor associated with skin autofluorescence after age (P<0.001) and diabetes mellitus (P<0.001) in multiple regression analysis. On univariate multinomial logistic regression analysis, every 1-U increase in skin autofluorescence was associated with a 7.43-fold (95% confidence intervals, 3.59–15.37; P<0.001) increased odds of having CACS ≥400 compared with those with zero CACS. Skin autofluorescence retained significance in predicting CACS ≥400 (odds ratio, 3.63; 95% confidence intervals, 1.44–9.18; P=0.006) when adjusting for age, sex, serum calcium, phosphate, albumin, C-reactive protein, lipids, blood pressure, estimated glomerular filtration rate, and intact parathyroid hormone but marginally lost significance when additionally adjusting for diabetes mellitus (odds ratio, 2.23; 95% confidence intervals, 0.81–6.14; P=0.1). Combination of diabetes mellitus and higher intact parathyroid hormone was associated with greater skin autofluorescence and CACS versus those without diabetes mellitus and having lower intact parathyroid hormone.

Conclusions—Tissue advanced glycation end product, as reflected by skin autofluorescence, showed a significant novel association with vascular calcification in chronic kidney disease. These data suggest that increased tissue advanced glycation end product may contribute to vascular calcification in chronic kidney disease and diabetes mellitus and warrant further experimental investigation. (Arterioscler Thromb Vasc Biol. 2014;34:00–00.)

Key Words: glycosylation end products, advanced • renal insufficiency, chronic • vascular calcification

Vascular calcification is a frequent complication in patients with diabetes mellitus and chronic kidney disease (CKD) and predicts an increased risk of mortality and cardiovascular events. Advanced glycation end products (AGE) represent a group of heterogeneous compounds that are formed through nonenzymatic glycation and oxidation of proteins and lipids after exposure to aldose sugars and frequently accumulate in diabetes mellitus and renal failure. The accumulation of AGE has been suggested to play an important role in inflammation and atherosclerosis. Recent experimental data showed that tissue AGE may induce calcification of vascular smooth muscle cells through the receptor for AGE (RAGE)/p38 mitogen-activated protein kinase signaling pathway. The skin autofluorescence reader is a recently developed noninvasive device that measured fluorophore of ultraviolet-A spectrum. Skin autofluorescence is determined from the ratio between the emission fluorescence in the wavelength range of 420 to 600 nm and the reflected excitation light with a wavelength range of 300 to 420 nm measured using a spectrometer and AGE reader software. In this wavelength band, the major contribution in fluorescence comes from fluorescent AGEs linked mostly not only to collagen but also to other proteins and lipids. Thus, skin autofluorescence is a group reactivity, reflecting total skin AGE pool rather than an individual fluorophore. Skin autofluorescence has been validated against AGE measurements from skin biopsy and shown to correlate reasonably well with skin AGE residues, including N-carboxymethyllysine, pentosidine, and N-carboxyethyllysine in patients with diabetes mellitus and in hemodialysis patients. Skin autofluorescence assessment is broadly applicable and has been shown to predict the development of microvascular
Table I in the online-only Data Supplement presents baseline characteristics of the study cohort. Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n=290</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60±10</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>162 (52)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>125 (43)</td>
</tr>
<tr>
<td>Chinese race, n (%)</td>
<td>290 (100)</td>
</tr>
<tr>
<td>Background atherosclerotic vascular disease, n (%)</td>
<td>56 (19.3)</td>
</tr>
<tr>
<td>Background coronary artery disease, n (%)</td>
<td>26 (9)</td>
</tr>
<tr>
<td>Background heart failure, n (%)</td>
<td>10 (3.4)</td>
</tr>
<tr>
<td>Background stroke, n (%)</td>
<td>28 (9.7)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9±4.5</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>204 (70.3)</td>
</tr>
<tr>
<td>Smoker</td>
<td>34 (11.7)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>52 (17.9)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>129±19</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>77±11</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>12.0±2.1</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>35.9±5.9</td>
</tr>
<tr>
<td>Serum albumin, g/dL</td>
<td>41.8±3.5</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate, ml/min per 1.73 m²</td>
<td>33.3±17.7</td>
</tr>
<tr>
<td>Serum calcium, mmol/L</td>
<td>2.35±0.10</td>
</tr>
<tr>
<td>Serum phosphate, mmol/L</td>
<td>1.22±0.25</td>
</tr>
<tr>
<td>Intact PTH, pmol/L</td>
<td>8.99 (6.15, 15.80)</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.84±2.00</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.51±1.00</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>4.65±1.04</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.68±0.89</td>
</tr>
<tr>
<td>High-sensitive C-reactive protein, mg/L</td>
<td>1.28±0.40</td>
</tr>
<tr>
<td>Total coronary artery calcium score*</td>
<td>41 (0.352)</td>
</tr>
<tr>
<td>Skin autofluorescence, AU</td>
<td>2.58±0.58</td>
</tr>
</tbody>
</table>

Data expressed as mean±SD unless specified otherwise. AU indicates arbitrary units; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and PTH, parathyroid hormone.

*Median (interquartile range).
Characteristics of Patients in Relation to Degree of Coronary Artery Calcification

Table 3 details the characteristics of patients in relation to the degree of CACS stratified into 4 groups, namely CACS =0, ≥1 to 99, ≥100 to 399, and ≥400. We observed a significant increasing trend in age (P<0.001), % of men (P<0.001), prevalence of diabetes mellitus (P<0.001), background atherosclerotic vascular disease (P<0.001), coronary artery disease (P<0.001), and heart failure (P<0.001) across the 4 groups of patients with increasing CACS. A significant increase in skin autofluorescence was observed across the 4 groups of patients with increasing CACS (P<0.001). Figure 2A shows the degree of coronary artery calcification in relation to tertiles of skin autofluorescence. Of the various biochemical parameters, a significant declining trend in eGFR (P=0.001) and low-density lipoprotein cholesterol (P=0.001) and an increasing trend

Table 2. Multiple Linear Regression Analysis of Skin Autofluorescence

<table>
<thead>
<tr>
<th>Variables</th>
<th>Model 1</th>
<th>P</th>
<th>Model 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increasing age, y</td>
<td>0.28</td>
<td>&lt;0.001</td>
<td>0.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.27</td>
<td>&lt;0.001</td>
<td>0.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive smoking history</td>
<td>0.16</td>
<td>0.006</td>
<td>0.16</td>
<td>0.006</td>
</tr>
<tr>
<td>Estimated GFR, mL/min per 1.73 m²</td>
<td>−0.17</td>
<td>0.004</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Male sex</td>
<td>−0.15</td>
<td>0.01</td>
<td>−0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>0.11</td>
<td>0.06</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>Intact PTH, pmol/L</td>
<td>…</td>
<td>…</td>
<td>0.19</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Covariates considered in model 1 include background coronary artery disease, systolic blood pressure, plasma phosphate, fasting glucose, plasma albumin, and high-sensitivity C-reactive protein. Model 2 includes all covariates in model 1 and intact parathyroid hormone (PTH). GFR indicates glomerular filtration rate.

Figure 1. A, Skin autofluorescence in relation to chronic kidney disease stages and diabetes mellitus status. Error bars indicate mean (95% confidence intervals [CIs]). B, Skin autofluorescence in relation to tertiles of intact parathyroid hormone (PTH). Error bars indicate mean (95% CIs). C, Skin autofluorescence in relation to diabetes mellitus status and tertiles of intact PTH, namely intact PTH ≤8.94 pmol/L (lower), between 6.95 and 12.50 pmol/L (middle), and ≥12.51 pmol/L (upper tertile). Error bars indicate mean (95% CIs).
in fasting glucose ($P<0.001$) and triglyceride ($P=0.015$) were observed across the 4 groups of patients with increasing CACS. No significant association was observed between plasma calcium, phosphate, and alkaline phosphatase with the degree of CACS. A trend toward increasing intact PTH was observed across the 4 groups of patients with increasing CACS but not reaching statistical significance ($P=0.1$). Figure 2B shows the prevalence of different degree of CACS in relation to tertiles of intact PTH and diabetes mellitus status.

### Multinomial Logistic Regression Analysis in Relation to CACS

Table 4 presents the univariate and multivariate multinomial logistic regression analysis of skin autofluorescence in relation to CACS stratified into groups, namely CACS $>1$ to 99, CACS $\geq 100$ to 399, and CACS $\geq 400$, with zero CACS as the reference group. In the univariate multinomial logistic regression analysis, every 1-U increase in skin autofluorescence was associated with a 2.13-fold (95% confidence intervals [CI],...
Table 4. Univariate and Multivariate Multinomial Logistic Regression Analysis of Skin Autofluorescence in Relation to the Degree of CACS

<table>
<thead>
<tr>
<th>CACS</th>
<th>Univariate Odds Ratio (95% Confidence Intervals)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACS 1–99</td>
<td>2.13 (1.07–4.22)</td>
<td>0.03</td>
</tr>
<tr>
<td>CACS 100–399</td>
<td>3.43 (1.62–7.29)</td>
<td>0.001</td>
</tr>
<tr>
<td>CACS ≥400</td>
<td>7.48 (3.59–15.37)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Coronary artery calcium score (CACS)=0 as reference group. Model 2, adjusting for age, sex, plasma calcium, phosphate, albumin, high-sensitivity C-reactive protein, systolic blood pressure, diastolic blood pressure, low-density lipoprotein cholesterol, and triglyceride other than age and sex, every 1-U increase in skin autofluorescence was associated with a 1.07-fold increased odds (95% CI, 1.62–7.29; P=0.001) and 7.43-fold (95% CI, 3.59–15.37; P<0.001) increased odds of having CACS ≥1 to 99, ≥100 to 399, and ≥400, respectively, compared with the reference group. Adjusting for age and sex, every 1-U increase in skin autofluorescence was associated with a 5.76-fold (95% CI, 2.45–13.51; P<0.001) increased odds of having CACS ≥400 compared with the reference group (model 1). In the stepwise multivariate multinomial logistic regression analysis additionally adjusting for plasma calcium, phosphate, albumin, high-sensitivity C-reactive protein, systolic and diastolic blood pressure, low-density lipoprotein cholesterol, and triglyceride other than age and sex, every 1-U increase in skin autofluorescence was associated with a 3.60-fold increased odds (95% CI, 1.43–9.07; P=0.006) of having CACS ≥400 compared with reference group (model 2). Additionally adjusting for eGFR did not reduce the association between skin autofluorescence and CACS ≥400 (odds ratio, 3.62; 95% CI, 1.42–9.22; P=0.007; model 3). Further adjusting for intact PTH did not reduce the association between skin autofluorescence and CACS ≥400 (odds ratio, 3.82; 95% CI, 1.47–9.90; P=0.006; model 4). However, further adjusting for fasting glucose reduced the association between skin autofluorescence and CACS ≥400 (odds ratio, 3.49; 95% CI, 1.34–9.09; P=0.01; model 5). The association between skin autofluorescence and CACS ≥400 lost significance (odds ratio, 2.21; 95% CI, 0.79–6.16; P=0.1) when additionally adjusting for diabetes mellitus (model 7; Table 4).

Receiver-Operating Characteristics Curve Analysis of Skin Autofluorescence in Predicting CACS

Using receiver-operator characteristics curve analysis, the area under the curve in predicting CACS ≥400 was 0.71 (95% CI, 0.65–0.76) for skin autofluorescence (P<0.0001) and was much higher compared with other biochemical parameters of CKD–mineral bone disease (Figure 3). The skin autofluorescence level that best predicted patients with CACS ≥400 was 2.76 AU with a sensitivity of 56.1% and a specificity of 78.1%. The positive predictive value associated with skin autofluorescence level of 2.76 was 43% and negative predictive value was 85.8%.

Discussion

To the best of our knowledge, this study is the first to demonstrate an important link between skin autofluorescence, reflecting tissue AGE accumulation, and CACS in patients with stage 3 to 5 CKD. Notably, the combination of diabetes mellitus and stage 5 CKD was associated with the highest skin autofluorescence and highest CACS. Skin autofluorescence retained independent, significant association with CACS ≥400 when adjusting for age, sex, blood pressure,
plasma calcium, phosphate, intact PTH, albumin, high-sensitivity C-reactive protein, low-density lipoprotein cholesterol, triglyceride, fasting glucose, and eGFR. However, the association was lost when additionally adjusting for diabetes mellitus. These data suggest that AGE may contribute to vascular calcification in CKD and diabetes mellitus. Skin autofluorescence has been shown to correlate well with skin level of AGE residues, including N-carboxymethyllysine, pentosidine, and N-carboxyethyllysine, and predict an increased mortality in hemodialysis patients and in patients with diabetes mellitus. Experimental studies showed that AGE is capable of inducing osteoblast-like differentiation and calcification of vascular smooth muscle cells through the RAGE/p38 mitogen-activated protein kinase signaling pathway. Similarly, another study showed an increased expression of RAGE and typical bone proteins, including osteopontin and alkaline phosphatase in conjunction with increased calcium accumulation in rat aortic vascular smooth muscle cells incubated with AGE in a time- and dose-dependent manner. These AGE-mediated changes in vascular smooth muscle cells were partially attenuated by a neutralizing antibody to RAGE. AGE has also been shown to accelerate vascular calcification through the RAGE/oxidative stress pathway in an animal model of diabetic vascular calcification. Thus, our current novel clinical observations add to the growing experimental evidence linking AGE to vascular calcification and support the involvement of tissue AGE in the process of vascular calcification in CKD and diabetes mellitus. In keeping with our findings, a previous study showed that plasma AGE (as denoted by pentosidine level) together with lipid peroxides (indicating oxidative stress) was linked to coronary artery calcification in dialysis patients. The other key finding is the important novel association between intact PTH and skin autofluorescence. Intact PTH displaced eGFR as the third most significant correlate of skin autofluorescence after age and diabetes mellitus. Notably, when stratifying patients into subgroups according to the presence or absence of diabetes mellitus and level of intact PTH, the combination of diabetes mellitus and intact PTH of the upper tertile was clearly associated with the highest skin autofluorescence and CACS. There is preliminary suggestion that AGE collagen cross-links was associated with disorders of bone mineralization in dialysis patients. Taken together, our findings demonstrate an important link between AGE, intact PTH, diabetes mellitus, and vascular calcification that warrants further elucidation.

As shown by our data and others, diabetes mellitus was one of the strongest determinants of skin autofluorescence. Hyperglycemia drives the nonenzymatic reaction between proteins and glucose in the Maillard reaction to form AGE. AGE may be formed during oxidative stress involving reactive carbonyl compounds. AGE may also accumulate as a result of ingestion of food with high AGE content or impaired renal clearance. In keeping with a number of studies showing an important relationship between worsening kidney function and increased skin autofluorescence, we observed a significant inverse association between eGFR and skin autofluorescence. Given the cross-sectional study design, it is currently not known whether an increased skin autofluorescence with worsening eGFR may be attributed to decreased renal AGE clearance or increased production through dicarbonyl and oxidative stress. Our results showed that the combination of diabetes mellitus and CKD was associated with the highest skin autofluorescence than either factor alone. Tissue AGE accumulation may further cross-link with proteins such as collagens and interact with RAGE, leading to activation of intracellular signaling pathways with cytokine release, inflammation, and tissue damage. RAGE has been implicated as playing a pathogenic role in CKD and cardiovascular disease. Skin autofluorescence is a strong and independent predictor of overall and cardiovascular mortality in hemodialysis patients and has been inversely correlated with circulating endothelial progenitor cells and positively associated with arterial stiffness in patients with end-stage renal disease. A recent systematic review also reported positive associations between skin autofluorescence and various diabetic complications, including nephropathy, neuropathy, and other macrovascular complications except retinopathy. In our current study, diabetes mellitus and skin autofluorescence showed strong associations with vascular calcification. More than 70% of patients with CACS ≥400 were diabetics. Skin autofluorescence was the highest among diabetic CKD patients with CACS ≥400. However, the association between skin autofluorescence and CACS was lost when additionally adjusting for diabetes mellitus. These findings provide important novel evidence that vascular calcification may underlie the important associations between increased skin autofluorescence with adverse overall and cardiovascular outcomes in patients with CKD and diabetes mellitus. There is evidence that skin autofluorescence may qualify as a better marker of cumulative metabolic stress and tissue AGE accumulation than circulating AGE levels and specifically reflects tissue AGEs, including pentosidine, N-carboxymethyllysine, and N-carboxyethyllysine. Preliminary analysis from our receiver-operator characteristics curve analysis also suggests that skin autofluorescence may be useful in predicting CACS ≥400.
In this study, age was one of the most important determinants of skin autofluorescence. The linear association between increasing chronological age and skin autofluorescence was previously reported in healthy individuals and other CKD cohorts. However, our study did not observe any significant relations between individual biochemical parameters of CKD–mineral bone disease, including serum calcium, phosphate, and alkaline phosphatase with vascular calcification. The exact explanation is not clear but may be explained by one-off time point sampling of various biochemical parameters that did not reflect cumulative burden of disordered mineral metabolism.

Our study has several limitations. First, we cannot make causal inferences based on the cross-sectional study design. Second, the current skin autofluorescence reader cannot be applied to patients with dark skin. However, this accounted for <1% in our study. Third, we did not have data on albuminuria.

In conclusion, our results provide important novel evidence linking skin autofluorescence, a proxy of tissue AGE, to vascular calcification in CKD and diabetes mellitus and support AGE playing a role in vascular calcification in CKD and diabetes mellitus that warrant further experimental investigation. Longitudinal study is needed to determine whether tissue AGE may predict progression of vascular calcification in CKD.

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We acknowledge Sharon Cheung for assisting in the study coordination and clinical data collection. We are indebted to all patients who participated in this study.

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Disclosure
Dr Wang has received speaker honoraria from Fresenius Kabi and Sanofi, grants from Sanofi, Baxter, and Abbvie, and has served as an advisory board member of Sanofi. The other authors report no conflicts.

References

**Significance**

This is the first clinical study to describe a novel relationship between skin autofluorescence, reflecting cumulative metabolic stress and tissue advanced glycation end product with vascular calcification in chronic kidney disease. Notably, the association between skin autofluorescence and coronary artery calcification outweighed other biochemical parameters of chronic kidney disease–mineral bone disease. Our study is also the first to demonstrate a novel association between intact parathyroid hormone and skin autofluorescence. Intact parathyroid hormone displaced estimated glomerular filtration rate as the third most significant factor associated with skin autofluorescence after age and diabetes mellitus in multiple regression analysis. The combination of diabetes mellitus and higher intact parathyroid hormone was associated with the highest skin autofluorescence and coronary artery calcium score in chronic kidney disease. Even though causal inferences cannot be made based on the cross-sectional study design, these several novel findings provide important evidence linking tissue advanced glycation end product to vascular calcification and disordered mineral metabolism in chronic kidney disease and pave way for experimental studies to elucidate exact underlying mechanisms. Longitudinal study is needed to determine whether higher tissue advanced glycation end product may predict more progression of vascular calcification.
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MATERIALS AND METHODS

Study Design

This is a prospective cross-sectional study conducted in a university teaching hospital and a major regional tertiary referral center in Hong Kong. The study protocol was approved by the institutional review board and ethics committee of the Hong Kong West Cluster. All patients provided written informed consent prior to study entry.

Study Subjects

Between May and December 2011, we randomly recruited 300 eligible CKD patients from the Renal Outpatient Clinic of the hospital. Inclusion criteria were patients with stages 3-5 CKD defined according to the National Kidney Foundation – Kidney Disease Outcomes Quality Initiative guidelines which classifies estimated glomerular filtration rate (eGFR) in the range from 30-59, 15-29 and <15 as stages 3, 4 and 5 CKD, respectively (1). We used the abbreviated Modification of Diet in Renal Disease equation to estimate GFR (2). Exclusion criteria were patients with underlying malignancy, chronic liver disease, systemic lupus erythematosus, chronic rheumatic heart disease, congenital heart disease and patients who refused to give consent.

Data Collection

We collected data on smoking history, diabetes, hypertension and medications on study entry. With a mercury sphygmomanometer, systolic and diastolic blood pressure was measured twice when patients presented to the center for study echocardiographic examination. Measurements were made on either arm after the patient was rested for 15 minutes and readings were averaged to give the final systolic and diastolic blood pressure.

Skin AF

Skin AF, as a measure of tissue AGE deposition was assessed on the right forearm in all study patients using the autofluorescence reader (AFR) device (DiagnOptics BV, Groningen, The Netherlands). Three measurements were taken and the average was calculated. Care was taken to avoid areas of skin that were tattooed or had cosmetics or sunscreen products applied. Patients with very dark or black or tanned skin were excluded from AF assessment as according to the manufacturer, the AFR and its software have not been validated in patients with skin reflection < 6%. If the ultraviolet
reflectance is below 6%, the AFR gives a warning that the signal is too low for valid results. All assessments were conducted by a single operator. The skin AF assessment is non-operator dependent.

**Multi-Slice Computed Tomography**

Plain multi-slice computed tomography scan was performed from pulmonary trunk bifurcation to base of the heart with a VCT 64 slice multi-detector computed tomography (General Electric, Milwaukee, WI, USA). Plain axial images were obtained with single breath-hold after a 5 second delay and electrocardiogram gating. Images were acquired using 120 kVp, 650 mAs, FOV of 25 cm, gantry rotation time 0.35 seconds, 0.625mm slice thickness & reconstructed slice thickness at 2.5 mm.

Calcium score analyses on coronary arteries were carried out on Advantage Window Workstation (Version 4.2). Semi-quantitative scores were estimated based on a section-by-section analysis of the computed tomography images. The total Agatston-Janowitz score was used as the scoring method. To be measurable in the Agatston-Janowitz score, the calcium plaque must reach a threshold of 130 HU and covers an area of at least 1 mm². Calcifications that were lower in attenuation or smaller in size were not included in the score. The score of each calcification was calculated by multiplying the area of the calcified plaque by an attenuation-weighting factor based on the highest HU value of the calcified plaque. A vessel score was the sum of all calcium scores of that vessel, and total calcium score was the total of all calcium score scores from all vessels of interest. All calcium scores were analyzed by a single experienced radiologist blinded to all clinical and biochemical details with intra-reader variability consistently below 5%. Patients were stratified into groups of increasing coronary artery calcium score (CACS), namely 0, ≥ 1-99, ≥ 100 – 399 and ≥ 400 (3).

**Biochemical measurements**

Fasting heparinized and EDTA blood samples were collected at baseline for measurement of plasma urea, creatinine, calcium, phosphorus, alkaline phosphatase, intact PTH, albumin, glucose, lipid profile and blood hemoglobin in a standard hospital biochemistry laboratory. High sensitivity C-reactive protein (hs-CRP) and intact PTH were measured by chemiluminescence immunoassays using IMMULITE® 1000 immunoassay system (Siemens Medical Solutions USA, Inc, Malvern, PA, USA).
Statistical analysis

Continuous data were expressed as mean ± SD or median (interquartile range), depending on the distribution of data, and categorical data as number (%). Between–group differences were compared using the one-way analysis of variance or Kruskal Wallis test for continuous data, depending on data distribution, or chi-square test for categorical data. Factors correlating with skin AF levels were evaluated using univariate and multiple linear regression analysis. Factors with P < 0.2 on univariate analysis were further considered in the multiple linear regression analysis using backward elimination strategy. Age and gender were included in the multiple linear regression analysis irrespective of their statistical significances in univariate analysis. We checked that all variables included in the multiple regression analysis had no significant multi-collinearities using the collinearity diagnostics. Univariate and stepwise multivariate multinomial logistic regression analysis were performed to evaluate the associations between skin AF levels and CACS ≥ 1-99, ≥ 100-399 and ≥ 400 (with 0 CACS as reference group). Adjustments were made in a stepwise fashion for age and sex, clinical and biochemical parameters including plasma calcium, phosphate, hs-CRP, systolic and diastolic blood pressure, low density lipoprotein (LDL)-cholesterol and triglyceride, eGFR, intact PTH, fasting glucose and diabetes. We performed receiver-operator-characteristics (ROC) curves analysis to investigate the value of skin AF in predicting CACS ≥ 400. The best skin AF cut-off level in predicting CACS ≥ 400 was derived from the ROC curves and was defined as the value that gave the best combination of sensitivity and specificity. A P value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using the SPSS software version 17.0 (SPSS, Inc., Chicago, Illinois, USA) and MedCalc Software version 7.50 (Mariakerke, Belgium).
REFERENCES

