

Skin Autofluorescence Measurement in Subclinical Atheromatous Disease: Results from the ILERVAS Project

Enric Sánchez¹, Àngels Betriu², Andree Yeramian¹, Elvira Fernández², Francesc Purroy³, Manuel Sánchez-de-la-Torre^{4, 5}, Reinald Pamplona⁶, Eva Miquel⁷, Mohsen Kerkeni⁸, Cristina Hernández^{9, 10}, Rafael Simó^{9, 10}, and Albert Lecube^{1, 10} on behalf of the ILERVAS project

ILERVAS Project: Marta Hernández¹, Ferran Rius¹, Dinora Polanco⁴, Ferran Barbé^{4, 5}, Gerard Torres^{4, 5}, Guillermo Suárez^{4, 5}, Manuel Portero-Otin⁶, Mariona Jové⁶, Laura Colàs-Campàs³, Ikram Benabdelhak³, Cristina Farràs⁷, Marta Ortega⁷, José Manuel Valdivielso², Marcelino Bermúdez-López², Montse Martínez-Alonso²

Enric Sánchez and Àngels Betriu contributed equally to this work.

Albert Lecube and Rafael Simó are joint senior authors.

¹Endocrinology and Nutrition Department. University Hospital Arnau de Vilanova. Obesity, Diabetes and Metabolism (ODIM) research group. IRBLleida. University of Lleida. Lleida, Catalonia, Spain.

²Unit for the Detection and Treatment of Atherothrombotic Diseases (UDETMA V&R). University Hospital Arnau de Vilanova. Vascular and Renal Translational Research Group. IRBLleida. University of Lleida. Lleida, Catalonia, Spain.

³Stroke Unit. University Hospital Arnau de Vilanova. Clinical Neurosciences Group. IRBLleida. University of Lleida. Lleida, Catalonia, Spain.

⁴Respiratory Department. University Hospital Arnau de Vilanova-Santa María. Translational Research in Respiratory Medicine. IRBLleida. University of Lleida. Lleida, Catalonia, Spain.

⁵Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

⁶Department of Experimental Medicine. IRBLleida. University of Lleida. Lleida, Catalonia, Spain.

⁷Borges Blanques Primary Health Care Unit. Lleida, Catalonia, Spain.

⁸Laboratory of Biochemistry, LR12ES05, Faculty of Medicine, University of Monastir. Monastir, Tunisia.

⁹Endocrinology and Nutrition Department, University Hospital Vall d'Hebron. Diabetes and Metabolism Research Unit, Vall d'Hebron Institut de Recerca (VHIR). Universitat Autònoma de Barcelona. Barcelona, Catalonia, Spain.

¹⁰Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

Aim: Advanced glycation end-products (AGEs) have been involved in the atherogenic process in the high-risk population. The goal of this study was to demonstrate that AGEs are related to subclinical atheromatous disease in subjects with low to moderate vascular risk.

Methods: A cross-sectional study in which 2,568 non-diabetic subjects of both sexes without cardiovascular disease were included. Subcutaneous content of AGEs was assessed by skin autofluorescence (SAF) and subclinical atheromatous disease was measured by assessing the atheromatous plaque burden in carotid and femoral regions using ultrasonography. In addition, serum pentosidine, carboxymethyl-lysine (CML) and AGE receptors (RAGE) were assessed in a nested case-control study with 41 subjects without plaque and 41 individuals subjects with generalized disease.

Results: Patients with atheromatous plaque had a higher SAF than those with no plaque (1.9 [1.7 to 2.3] vs. 1.8 [1.6 to 2.1] arbitrary units (AU), $p < 0.001$). The SAF correlated with the total number of affected regions ($r = 0.171$, $p < 0.001$), increasing progressively from 1.8 [1.6 to 2.1] AU in those without atheromatous disease to 2.3 [1.9 to 2.7] AU in patients with ≥ 8 plaques ($p < 0.001$). A correlation was also observed between SAF and the total plaque area ($r = 0.113$, $p < 0.001$). The area under the Receiver Operating Characteristic curve was 0.65 (0.61 to 0.68) for identifying male subjects with atheromatous disease. The multivariable logistic regression model showed a significant and independent association between SAF and the presence of atheromatous disease.

However, no significant differences in serum pentosidine, CML, and RAGE were observed.

Conclusions: Increased subcutaneous content of AGEs is associated with augmented atheromatous plaque burden. Our results suggest that SAF may provide clinically relevant information to the current strategies for the evaluation of cardiovascular risk, especially among the male population.

Key words: Advanced glycation end-products, Atheromatous plaque burden, Cardiovascular risk, Skin autofluorescence

Introduction

The natural progression of atherosclerosis involves a long silent period, and the advanced stages of the disease are frequently not detected until cardiovascular ischemic events occur¹. For this reason, the assessment of classical determinants of atherosclerosis, such as high blood pressure, atherogenic dyslipidemia, smoking habit, and type 2 diabetes has well-recognized limitations, particularly in lower-risk groups such as young people and women^{2, 3}. In addition, the identification of subclinical atherosclerosis may help physicians to identify vulnerable subjects at increased risk of cardiovascular disease (CVD) and develop strategies to arrest disease progress^{4, 5}.

The advanced glycation end-products (AGEs) constitute a complex group of compounds formed by the slow non-enzymatic glycation of proteins, lipids, and nucleic acids, of which about 20 have been identified to date⁶. The AGEs have been related to the appearance and progression of vascular changes such as increased permeability and thickening and stiffness of the arterial wall, resulting in the development of atheromatous plaques decreasing luminal space⁷⁻⁹. However, whether or not the magnitude of AGEs deposition in vascular walls is related to the main characteristics of atheromatous disease, such as the number of affected regions, plaque location, and the total area of the atheromatous plaques remain to be elucidated. To shed light on this issue, we performed a cross-sectional study using the non-invasive assessment of tissue accumulation of AGEs through skin autofluorescence (SAF) in 2,568 subjects without a previous history of vascular disease who had undergone bilateral carotid and femoral ultrasound exploration in the setting of the ILERVAS project¹⁰.

In addition, the association between circulating

AGEs and vascular disease has been previously evaluated with opposing results¹¹⁻¹³. Therefore, we also designed a nested case-control study addressed to evaluate the serum concentration of pentosidine, carboxymethyl-lysine (CML), and AGEs receptors (RAGE) in 41 subjects without plaque and 41 patients with generalized disease.

Methods

Design of the Study and Description of the Study Population

Cross-Sectional Study

A total of 2,568 Caucasian subjects were enrolled between July 2015 and December 2017 from primary health centers in an ongoing prospective study aimed at evaluating the presence of asymptomatic CVD in the province of Lleida, Spain (ILERVAS Project; ClinTrials.gov Identifier: NCT03228459)¹⁰.

The following inclusion criteria were used: men and women aged between 45 and 70 years old, without any history of vascular disease (ischemic heart disease, stroke, or peripheral arteriopathy), and at least one traditional cardiovascular risk factor (hypertension, dyslipidemia, a body mass index (BMI) ≥ 30 kg/m², smoking, or a first-degree family member with premature cardiovascular disease). Clinical information was obtained from an electronic database (Information System for the Development of Research in Primary Care, SIDIAP) that contains anonymized, longitudinal data from the 31 primary care centers pertaining to the Catalan Health Institute in the province of Lleida, Spain¹⁴. The exclusion criteria comprised of the presence of chronic kidney disease, active neoplasia, a life expectancy of fewer than 18 months, darker skin color (Fitzpatrick classes >5), and pregnant women. Subjects with type 1 and type 2 diabe-

Address for correspondence: Albert Lecube, Endocrinology and Nutrition Department, University Hospital Arnau de Vilanova Obesity, Diabetes and Metabolism (ODIM) research group, IRBLleida Avda. Rovira Roure 80. 25198-Lleida, Spain E-mail: alecube@gmail.com

Received: October 9, 2018 Accepted for publication: January 10, 2019

Copyright©2019 Japan Atherosclerosis Society

This article is distributed under the terms of the latest version of CC BY-NC-SA defined by the Creative Commons Attribution License.

tes, major risk factors for cardiovascular disease were also excluded¹⁵.

For all subjects, data were available regarding both SAF and vascular carotid and femoral ultrasound examinations. Anthropometrical data were obtained by standardized protocols. Smoking status (never/current/ex-smoker) was recorded. Smokers who stopped smoking ≥ 1 year prior to recruitment were considered ex-smokers.

The prescribed antihypertensive and lipid-lowering treatments were extracted from prescription- and pharmacy-invoicing databases provided by the Cat-Salut (Catalan Health Service), which are incorporated yearly into the SIDIAP database. Antihypertensive medication agents included angiotensin-converting enzyme inhibitors, diuretics, aldosterone receptor antagonist type II, beta-blockers, calcium antagonists, and other antihypertensives. Lipid-lowering drugs included statins, fibrates, ezetimibe, and omega-3 fatty acids. In addition, the use of anticoagulant or antiplatelet agents was also recorded.

Nested Case-Control Study

Serum concentrations of pentosidine, CML, and RAGE were assessed in a subgroup of 41 subjects with generalized atheromatous subclinical disease (≥ 4 affected territories with plaque) matched (1:1) to 41 controls without plaque carefully matched by gender, age (± 4 years), the BMI (± 3 kg/m²) and total cholesterol (± 30 mg/dL). The inclusion and exclusion criteria were the same as in the cross-sectional study.

Assessment of Atheromatous Plaque Burden

Bilateral carotid (common artery, bifurcation, internal, and external) and femoral (common and superficial) areas were explored. The images were obtained by trained sonographers using an ultrasonic Doppler Ultrasound Vivid-I (General Electrics Healthcare, Waukesha, WI) equipped with a linear transducer broadband linear 12L-RS that operates at frequencies between 5–13 MHz. Standardized and validated scanning and reading protocols were used to decrease inter-operator variability and type 2 errors¹⁶. To evaluate intraobserver reliability, a total of 20 individuals were measured three to five times on diverse days. A k-coefficient of one was obtained for plaque assessment, demonstrating excellent intraobserver reliability. The readers were unaware of the patients' clinical histories.

Subclinical atheromatosis was defined as the presence of at least one plaque in any of the 12 assessed areas¹⁷. A plaque was defined as a focal intima-media thickness ≥ 1.5 mm protruding in the lumen¹⁸. Participants were classified as having focal (1

region), intermediate (2 to 3 regions), or generalized (4 to 12 regions) atheromatous disease. The carotid plaque area was measured, and the total plaque area (cm²) was defined as the sum of the area of each plaque within the same subject^{19, 20}.

Measurement of Skin Autofluorescence

SAF was measured using the AGE ReaderTM device (DiagnOptics Technologies, Groningen, The Netherlands); a fully automated non-invasive tool that measures AGEs deposition in the forearm using an Ultraviolet-A spectrum²¹. The mean value of three readings (arbitrary units: AU) was recorded. Skin areas that were tattooed or colored with cosmetics, were heavily freckled, or had vessels near to the surface of the skin were avoided. A single device, maintained and calibrated by the manufacturer following their recommendations, was used for all measurements. Repeated SAF measurements with the AGE ReaderTM device taken over a single day showed an overall Altman error percentage of 5.03%, and intra-individual seasonal variance showed an Altman error percentage of 5.87%²¹.

Evaluation of Lipid Profile and Glycated Hemoglobin

Total cholesterol (mg/dl) concentrations were obtained in the entire population included in the study from a non-fasting dried capillary blood testing (fingertip puncture) using the Reflotrons[®] Plus system (Roche). Determination of the complete lipid profile [high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides] was determined only in subjects in which total cholesterol was ≥ 200 mg/dL and fasted for 6 hours or total cholesterol ≥ 250 mg/dL regardless of fasting hours. As the presence of type 2 diabetes was an exclusion criterion in the ILERVAS project, data of fasting blood glucose is not available in our study. However, the glycated hemoglobin (HbA1c) test was performed to all participants using a point-of-care device [Cobas B 101[®] (Roche Diagnostics) system].

Laboratory Assessment of Serum AGEs

In addition to previous determinations, a venous blood sample was collected from the antecubital vein, separated by centrifugation (2.000 g at 4°C for 20 min) and frozen at -80°C for batched storage and analysis. Samples were obtained with support by IRLleida Biobank (B.0000682) from Plataforma Biobancos (PT13/0010/0014). Serum concentrations of pentosidine, CML, and the soluble form of RAGE were determined in duplicate by an enzyme-linked immunosorbent assay (ELISA) kit provided by MyBioSource (catalog number MBS036744,

MBS721957 and MBS724049, respectively; MyBioSource, Inc. San Diego, CA, USA), according to the manufacturer's instructions. Briefly, samples were added to a microtiter plate that has been previously pre-coated with a specific antibody against each human AGE (RAGE, CML, or pentosidine). After incubation and several washing steps, plates were incubated with a secondary antibody conjugated to horseradish peroxidase and OD450 nm was determined using an ELISA plate reader (Source Bioscience, Nottingham, UK). Intra-assay and inter-assay precision coefficient of variation <15% for all analytes.

Statistical Analysis

The normal distribution of the variables was evaluated using the Shapiro–Wilk test. Given its skewed distribution, quantitative data are expressed as the median [interquartile range]. Comparisons between groups were made using the Mann–Whitney *U* test for quantitative variables, and the Pearson's chi-squared for categorical variables. The relationship between continuous variables was assessed by the Spearman correlation test.

The accuracy of SAF as a measurement of interest in discriminating diseased subjects (patients with ≥ 1 plaque) from cases without atheromatous disease was evaluated using a Receiver Operating Characteristic (ROC) curve analysis with a complete sensitivity/specificity report and calculating Youden *J* statistic.

A multivariable logistic regression model with the enter method was used to explore the variables that were independently associated with the presence of atheromatous plaque. The independent variables included in the analysis were SAF and well-established cardiovascular risk factors such as gender, age, smoking status, systolic blood pressure, lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides), together with HbA1c and antihypertensive or lipid-lowering treatment. The calibration and discrimination of the logistic model were evaluated using the test of fit Hosmer–Lemeshow and the area under the curve, respectively. All “*p*” values were based on a two-sided test of statistical significance. Significance was accepted at the level of *p* value <0.05. The analyses were performed using SPSS statistical package (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA).

Ethics Statement

All participants signed an informed consent, and the study was approved by the Arnau de Vilanova University Hospital ethics committee (CEIC-1410) and was conducted according to the ethical guidelines of the 1964 Helsinki Declaration and its later amendments.

Results

The prevalence of subclinical atheromatous disease in the study population was 70.4%: 21.8% restricted to carotid regions, 33.2% restricted to femoral regions, and 45.0% with plaques in both regions. The main clinical characteristics and metabolic data according to the presence of plaque are shown in **Table 1**. Asymptomatic atherosclerosis disease was higher in active smokers and in men, and increased with age, blood pressure levels, and LDL cholesterol concentration. In addition, patients with subclinical atheromatous disease showed a higher SAF than subjects without disease (1.9 [1.7 to 2.3] vs. 1.8 [1.6 to 2.1] AU, $p < 0.001$). SAF was also higher when both carotid and femoral regions were affected (2.0 [1.7 to 2.3] AU) than when the disease was restricted to carotid (1.9 [1.7 to 2.2] AU, $p = 0.008$) or femoral (1.9 [1.7 to 2.2] AU, $p = 0.004$) regions. Finally, SAF increased progressively from patients without plaque (1.8 [1.6 to 2.1] AU) to those with generalized (2.0 [1.8 to 2.4] AU) subclinical atherosclerosis ($p < 0.001$) (**Fig. 1A**). Subjects with ≥ 8 affected regions showed the higher SAF (2.3 [1.9 to 2.7] AU). In the univariate analysis, a positive correlation between SAF and the number of affected regions was observed ($r = 0.171$, $p < 0.001$).

The total plaque area was 0.51 [0.21 to 1.07] cm², ranging from 0.13 [0.09 to 0.23] in patients with only one plaque to 1.41 [0.90 to 2.12] cm² when the disease was generalized (**Fig. 1B**). A positive significant correlation was also observed between SAF and the total plaque area ($r = 0.113$, $p < 0.001$).

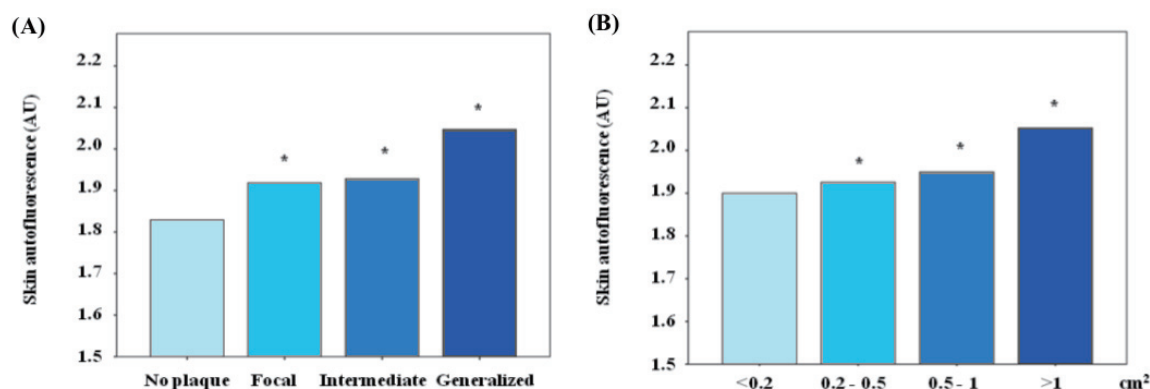
The ROC analysis revealed that the best cut-off point for SAF (combining sensitivity plus specificity) for identifying patients with a subclinical atheromatous disease was 2.05 AU in the entire population. At this point, the area under the ROC was 0.59 (0.57 to 0.61). This value increased to 0.65 (0.61 to 0.68) with a sensitivity of 55.6% and a specificity of 66.9% when the ROC analysis was restricted to men, and the cut-off point was reduced to 1.85 AU (**Fig. 2**). At this point, the percentage of subjects with any plaque increased from 70.6% among those with a SAF < 1.85 AU to 85.9% among those with a SAF ≥ 1.85 AU ($p < 0.001$). This data indicates more than a 2-fold increased risk of the presence of an atheromatous plaque (mean difference 2.5 [95% confidence interval (CI) 1.9 to 3.4]; $p < 0.001$) in comparison with subjects with lower SAF values. In addition, the total plaque area was higher among men with a SAF ≥ 1.85 AU in comparison with a lower SAF amount (0.81 [0.39 to 1.50] vs. 0.58 [0.24 to 1.15] cm², $p < 0.001$).

The multivariable logistic regression model

Table 1. Main clinical characteristics and metabolic data of the study population according to the presence of at least one atheromatous plaque

	Subjects without plaque	Subjects with ≥ 1 plaque	<i>p</i> value
<i>n</i>	760	1808	-
Women, <i>n</i> (%)	488 (64.2)	825 (45.6)	<0.001
Age (years)	56 [52 to 61]	58 [53 to 63]	<0.001
Dyslipidemia, <i>n</i> (%)	347 (45.7)	965 (53.5)	<0.001
Total Cholesterol (mg/dL)	198 [178 to 224]	203 [180 to 229]	0.015
LDL Cholesterol ¹ (mg/dL)	141 [125 to 154]	144 [129 to 163]	0.017
HDL Cholesterol ¹ (mg/dL)	58 [48 to 68]	54 [45 to 66]	0.015
Triglycerides ¹ (mg/dL)	124 [92 to 170]	135 [129 to 163]	0.001
Lipid-lowering agents, <i>n</i> (%)	98 (12.9)	349 (19.3)	<0.001
Blood hypertension, <i>n</i> (%)	257 (33.9)	782 (43.3)	<0.001
Systolic BP (mm Hg)	127 [116 to 138]	131 [121 to 143]	<0.001
Diastolic BP (mm Hg)	80 [74 to 87]	82 [76 to 89]	<0.001
Antihypertensives, <i>n</i> (%)	215 (28.3)	625 (34.6)	0.002
Obesity (BMI ≥ 30 kg/m ²), <i>n</i> (%)	229 (30.2)	517 (28.6)	0.437
BMI (kg/m ²)	28.7 [25.6 to 31.6]	28.6 [25.9 to 31.8]	0.563
Former smoker, <i>n</i> (%)	591 (27.7)	1455 (31.1)	<0.001
Current smoker, <i>n</i> (%)	407 (19.1)	1545 (33.0)	<0.001
First-degree family member with premature CVD, <i>n</i> (%)	81 (10.7)	202 (11.2)	0.702
Anticoagulants/Antiplatelets, <i>n</i> (%)	15 (2.0)	57 (3.2)	0.098
HbA1c (%)	5.5 [5.3 to 5.7]	5.5 [5.3 to 5.8]	0.006
eGFR (mL/min per 1.73 m ²)	96.6 [85.5 to 102.7]	95.2 [84.9 to 101.9]	0.063
SAF (AU)	1.8 [1.6 to 2.1]	1.9 [1.7 to 2.3]	<0.001

Data are median [interquartile range] *n* (percentage); BMI: body mass index; LDL: low density lipoprotein; ¹: determination was done in cases in which total cholesterol was ≥ 200 mg/dL and fast for 6 hours or total cholesterol ≥ 250 mg/dL regardless of fasting hours; eGFR: estimated glomerular filtration rate estimated according the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation; BP: blood pressure; BMI: body mass index; CVD: cardiovascular disease; HbA1c: glycated hemoglobin; SAF: skin autofluorescence.

**Fig. 1.** Skin autofluorescence according to the affected territories with atheromatous disease (A) and the quartiles of total plaque area (B)

Participants in A) were defined as having focal (1 territory), intermediate (2 to 3 territories), or generalized (4 to 12 territories) atheromatous disease. SAF: skin autofluorescence; AU: arbitrary units; * $p < 0.001$. Patients with focal, intermediate and generalize atheromatous disease showed a significantly higher SAF than subjects without plaque ($p < 0.001$, for all). Participants in B) were defined by quartiles of total plaque area. Patients in the second, third and fourth quartile showed a significantly higher SAF than subjects in the first quartile ($p < 0.001$, for all).

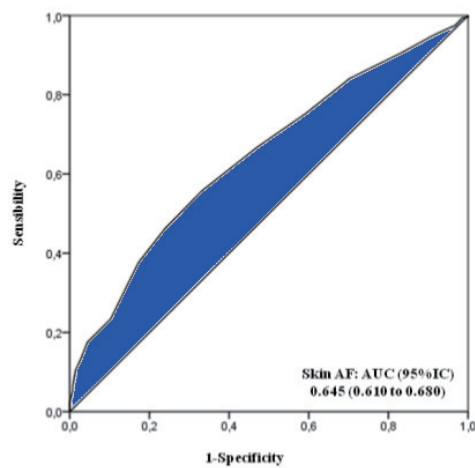


Fig. 2. ROC curve analysis to evaluate the accuracy of skin autofluorescence in presence of subclinical atheromatous disease from normal cases, together with a sensitivity / specificity report for the male population

AF: autofluorescence; AUC: area under the ROC curve. The total area under the ROC curve was interpreted following the following guidelines: 0.9 to 1.0, excellent; 0.8 to 0.9, good; 0.7 to 0.8, fair; 0.6 to 0.7, poor, and 0.5 to 0.6, not useful.

showed that older age, male gender, and current smoker followed by SAF were independent risk factors (all $p < 0.001$) for atheromatous disease in the entire population (**Table 2**). In fact, the risk of having atheroma plaque increases 1.8 times for each SAF unit. Systolic blood pressure and LDL cholesterol also appeared to be independently associated with atheromatous disease, but with a lower odds ratio than the previous factors. When males were evaluated alone, the risk of having any atheroma plaque increases 4.11 times for each SAF unit (**Table 3**).

Finally, no differences in serum concentrations of pentosidine, CML, or RAGE were observed among the 82 patients with and without generalized atheromatous disease (**Table 4**). In addition, SAF failed to show a significant correlation with CML ($r = 0.036$, $p = 0.791$), pentosidine ($r = 0.140$, $p = 0.284$) or RAGE ($r = -0.072$, $p = 0.769$) in this small subgroup of participants.

Table 2. The multivariable logistic regression model for presence of atheromatous disease and its variables associated including the entire population of the study

Atheromatous disease	OR (95% CI)	<i>p</i> value
Gender		
Women	Ref.	
Men	3.27 (2.15 to 4.97)	<0.001
Age (years)	1.11 (1.07 to 1.15)	<0.001
Smoking status		
Never	Ref.	
Former	2.03 (1.37 to 3.02)	<0.001
Current	4.32 (2.67 to 6.98)	<0.001
Skin autofluorescence (AU)	1.87 (1.23 to 2.86)	0.004
HbA1c (%)	1.39 (0.84 to 2.33)	0.202
Systolic blood pressure (mm Hg)	1.02 (1.01 to 1.03)	0.003
Antihypertensive treatment		
No	Ref.	
Yes	1.01 (0.69 to 1.49)	0.961
Total Cholesterol (mg/dL)		
< 200	Ref.	
≥ 200	1.53 (0.33 to 7.07)	0.588
LDL Cholesterol (mg/dL)	1.01 (1.00 to 1.01)	0.042
HDL Cholesterol (mg/dL)	1.01 (0.99 to 1.02)	0.239
Triglycerides (mg/dL)	1.00 (0.99 to 1.05)	0.138
Lipid-lowering agents		
No	Ref.	
Yes	0.70 (0.41 to 1.18)	0.175
Test of fit Hosmer–Lemeshow		0.118
Area under de ROC curve	0.76 (0.70 to 0.81)	<0.001

Table 3. Independent variables significantly associated with the presence of atheromatous disease in the multivariable logistic regression model according to gender

	OR (95% CI)	<i>p</i> value
Atheromatous disease in male		
Age (years)	1.07 (1.01 to 1.14)	0.025
Smoking status		
Never	Ref.	
Former	1.95 (1.01 to 3.79)	0.050
Current	3.48 (1.63 to 7.42)	0.001
Skin autofluorescence (AU)	4.11 (1.71 to 9.87)	0.002
Test of fit Hosmer–Lemeshow	-	0.522
Area under de ROC curve	0.77 (0.71 to 0.82)	<0.001
Atheromatous disease in female		
Age (years)	1.12 (1.08 to 1.17)	<0.001
Smoking status		
Never	Ref.	
Former	2.13 (1.27 to 3.57)	0.004
Current	5.02 (2.64 to 9.53)	<0.001
Systolic blood pressure (mm Hg)	1.02 (1.00 to 1.03)	0.012
LDL Cholesterol (mg/dL)	1.01 (1.00 to 1.01)	0.029
Test of fit Hosmer–Lemeshow	-	0.392
Area under de ROC curve	0.73 (0.68 to 0.77)	<0.001

The independent variables included in the analysis were SAF and well-established cardiovascular risk factors such as gender, age, smoking status, systolic blood pressure, lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides), together with HbA1c and antihypertensive or lipid-lowering treatment.

Table 4. Main clinical characteristics and serum concentration of advanced glycation end-products and its receptor of the nested case-control study according to the presence of generalized atheromatous disease

	Subjects without plaque	Subjects with plaque and generalized atheromatous disease	<i>p</i> value
<i>n</i>	41	41	
Women, <i>n</i> (%)	14 (34.1)	15 (36.6)	1.000
Age (years)	59 [55 to 63]	60 [55 to 65]	0.656
BMI (kg/m ²)	28.0 [25.9 to 29.9]	28.0 [26.5 to 30.5]	0.597
Total cholesterol (mg/dL)	210 [189 to 228]	202 [183 to 222]	0.358
Pentosidine (ng/mL)	25.8 [8.7 to 54.2]	21.9 [8.9 to 42.8]	0.842
CML (ng/mL)	105.0 [64.4 to 121.6]	109.8 [85.6 to 143.1]	0.070
AGEs receptor (ng/mL)	0.044 [0.018 to 0.142]	0.039 [0.023 to 0.066]	0.531
SAF (AU)	1.8 [1.6 to 2.1]	2.1 [1.9 to 2.4]	<0.001

BMI: body mass index; CML: carboxymethyl-lysine; AGEs: advanced end glycation products; SAF: skin autofluorescence; AU: arbitrary units.

Discussion

To the best of our knowledge, this is the first study in which SAF has been evaluated as a marker of subclinical atheromatous disease in a large cohort of subjects without a history of cardiovascular disease. In

our cohort of middle-aged asymptomatic subjects, a close relationship between SAF and the atheromatous plaque burden (the presence of plaque, the number of affected territories, and the total area of atheromatous plaque) has been observed.

The prevalence of subclinical atheromatous dis-

ease in our study was 70.4% compared with the 63% described in the middle-aged asymptomatic sample without cardiovascular disease from the Progression of Early Subclinical Atherosclerosis study²²). These studies reveal that the presence of atheromatous plaque in asymptomatic subjects is a common condition that deserves particular attention in the prevention of cardiovascular outcomes. Moreover, when nearly one-third of participants from the PESA study classified at low risk by traditional scales (i.e., Framingham Heart Study and the European Systematic Coronary Risk Evaluation) had intermediate or generalized atheromatous disease²²).

The present study also shows that when the screening of atheromatous disease was limited to carotid arteries, we failed to detect at least 12% of patients with disease in femoral regions. This finding is in concordance with data from the Carotid-Femoral Ultrasound Morphology and Cardiovascular Events study, which showed that scanning only carotid or only femoral regions predicted 15% and 13% fewer events than examining both territories in a 10-year follow-up²³).

Previous population studies, such as the Multi-ethnic Study of Atherosclerosis, the US High Risk Plaque Study, and the Northern Manhattan Study established robust associations between cardiovascular events, subclinical carotid and coronary disease^{4, 5, 20, 24}). Similarly, in middle-aged Spanish men from the Aragon Workers' Health Study, subclinical carotid and femoral plaques were associated with coronary artery calcium score²⁵), a well-recognized risk factor of coronary outcomes²⁶). In this setting, our data shows that SAF is a useful tool for helping clinicians to identify individuals at risk of developing cardiovascular disease.

Some characteristic effects of AGEs on plaque formation have been previously described, such as increased oxidative stress, modifications of collagen and other proteins that constitute the extracellular matrix of the arterial wall and the basement membrane, the promotion of LDL trapping in the sub-endothelial compartment, and the increment of vascular stiffness²⁷⁻³¹). Moreover, the binding of AGEs to their receptors activates nuclear factor kappa B, among other pro-oxidant and pro-inflammatory pathways^{32, 33}). Therefore, the role of AGEs favors the formation of atheromatous plaques causing widespread damage to arteries through the upregulation of inflammation, oxidative stress, and the cross-linking of collagen and other proteins of the arterial wall. In addition, AGEs also could act as triggers from stable to rupture-prone atheromatous plaques⁹).

Previous studies have shown that SAF levels increase proportionally with the number of compo-

nents of the metabolic syndrome, as well as with lower HDL cholesterol³⁴). In addition, SAF assessment has been associated with coronary and peripheral artery disease³⁵⁻³⁷). Moreover, the added clinical value of SAF is supported by data that reflects its measurement as a strong predictor of carotid atherosclerosis, long-term cardiovascular complications, and mortality in populations with increased cardiovascular risk, such as patients with type 1 diabetes and renal disease³⁸⁻⁴²).

As atheromatous disease is a diffuse process, the measurement of the total plaque area seems to be a more reliable approach to the patient at risk⁴³). It is worth mentioning that our study demonstrates a significant and independent association between SAF and total area plaque.

The association between circulating AGEs and both all-cause mortality and cardiovascular mortality has been previously assessed in moderate-sized epidemiological studies focused on healthy aging population, disabled older women, and patients with type 1 diabetes^{11, 44-47}). In this regard, in an adult population of men and women aged 65 and older, serum CML was associated with an increased incidence of coronary heart disease and stroke, independent of traditional atherosclerosis risk factors¹¹). However, Hanssen et al. showed that plasma AGE measurements were not different when comparing individuals with and without cardiovascular disease in participants from two Dutch cohort studies comprising 1,291 subjects with various degrees of impaired glucose metabolism¹²). Similarly, in a modest-sized cohort with chronic kidney disease subjects, no association between CML and incident CVD was detected¹³). Our data also failed to find any difference between circulating AGEs as well as its receptors with the presence of subclinical atheromatous disease, together with a lack of correlation between those serum markers and SAF. This result could be owing to the fact that circulating AGEs are rapidly broken down to AGE peptides or free AGEs, which are excreted through the kidney, thus having a fast turnover^{6, 48}). By contrast, AGEs linked to proteins from subcutaneous tissue have a slow turnover, thus reflecting a decades-long accumulation.

There are some potential limitations that must be highlighted in our study. First, as an observational and cross-sectional study, a causal relationship between SAF or circulating AGEs could not be established. Second, SAF could be unreliable in subjects with dark skin due to excessive light absorption. We overcame this limitation by selecting only Caucasian subjects and excluding those with medium brown skin. Third, the lack of statistical power of our nested study because of the small sample size assessed (82 subjects from the 2,568 in whom skin autofluorescence and

bilateral carotid and femoral areas were explored). Therefore, the data are not definitive but rather hypothesis-generating in nature, and larger studies will be needed to confirm our findings. In addition, as we enrolled subjects with generalized atheromatous disease, the generalizability to the overall population with at least one atheromatous plaque is limited.

Conclusion

There is a clear need to identify subjects at increased risk of CVD. Generally, clinicians have used global risk scores that combine multiple traditional cardiovascular risk factors to classify patients. Our study shows that SAF is associated with an increased atherosclerotic burden especially in men, and may provide clinically relevant information to the standard cardiovascular risk assessment strategies.

Acknowledgments

The authors would also like to thank Fundació Renal Jaume Arnó, Eva Castro, Virtudes María, Marta Elias, Teresa Vidal, M^a del Valle Peña, Cristina Dominguez, Noemi Nova, Alba Prunera, Núria Sans, Meritxell Soria, and the Primary Care teams from Lleida for recruiting subjects and their efforts in the accurate development of the ILERVAS project.

Funding Source

This work was supported by grants from the Diputació de Lleida, Instituto de Salud Carlos III (Action Plan II14//00008), Fundación de la Sociedad Española de Endocrinología y Nutrición (FSEEN), Esteve Laboratory, Generalitat de Catalunya (2017SGR696 and SLT0021600250), IRBLleida Biobank (B.0000682) and Plataforma Biobancos PT13/0010/0014. CIBER de Diabetes y Enfermedades Metabólicas Asociadas and CIBER de Enfermedades Respiratorias are initiatives of the Instituto de Salud Carlos III. These organizations had no role in study design, the collection, analysis and interpretation of data, report writing, or the decision to submit the article for publication. The authors declare that there are no relationships with industry relevant to this manuscript.

Conflicts of Interests

The authors declare that there is no conflict of interest.

References

- 1) Vilahur G, Badimon J, Bugiardini R, Badimon L: Perspectives: the burden of cardiovascular risk factors and coronary heart disease in Europe and worldwide. *Eur Heart J Supplements*, 2014; 16: A7-A11
- 2) McMahan CA, Gidding SS, Viikari JS, Juonala M, Kähönen M, Hutri-Kähönen N, Jokinen E, Taittonen L, Pietikäinen M, McGill HC Jr, Raitakari OT: Association of pathobiologic determinants of atherosclerosis in youth risk score and 15-year change in risk score with carotid artery intima-media thickness in young adults (from the Cardiovascular Risk in Young Finns Study). *Am J Cardiol*, 2007; 100: 1124-1129
- 3) Berry JD, Liu K, Folsom AR, Lewis CE, Carr JJ, Polak JF, Shea S, Sidney S, O'Leary DH, Chan C, Lloyd-Jones DM: Prevalence and progression of subclinical atherosclerosis in younger adults with low short-term but high lifetime estimated risk for cardiovascular disease: the Coronary Artery Risk Development in Young Adults Study and Multi-Ethnic Study of Atherosclerosis. *Circulation*, 2009; 119: 382-389
- 4) Zavodni AE, Wasserman BA, McClelland RL, Gomes AS, Folsom AR, Polak JF, Lima JA, Bluemke DA: Carotid artery plaque morphology and composition in relation to incident cardiovascular events: the Multi-Ethnic Study of Atherosclerosis (MESA). *Radiology*, 2014; 271: 381-389
- 5) Gibson AO, Blaha MJ, Arnan MK, Sacco RL, Szklo M, Herrington DM, Yeboah J: Coronary artery calcium and incident cerebrovascular events in an asymptomatic cohort: the MESA study. *JACC Cardiovasc Imaging*, 2014; 7: 1108-1115
- 6) Ajith TA, Vinodkumar P: Advanced glycation end products: association with the pathogenesis of diseases and the current therapeutic approaches. *Curr Clin Pharmacol*, 2016; 11: 118-127
- 7) Vlassara H, Fuh H, Donnelly T, Cybulsky M: Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol Med*, 1995; 1: 447-456
- 8) Ishibashi Y, Matsui T, Maeda S, Higashimoto Y, Yamagishi S: Advanced glycation end products evoke endothelial cell damage by stimulating soluble dipeptidyl peptidase-4 production and its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. *Cardiovasc Diabetol*, 2013; 12: 125
- 9) Hanssen NM, Wouters K, Huijberts MS, Gijbels MJ, Sluimer JC, Scheijen JL, Heeneman S, Biessen EA, Daemen MJ, Brownlee M, de Kleijn DP, Stehouwer CD, Pasterkamp G, Schalkwijk CG: Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype. *Eur Heart J*, 2014; 35: 1137-1146
- 10) Betriu À, Farràs C, Abajo M, Martínez-Alonso M, Arroyo D, Barbé F, Buti M, Lecube A, Portero M, Purroy F, Torres G, Valdivielso JM, Fernández E: Randomised intervention study to assess the prevalence of subclinical vascular disease and hidden kidney disease and its impact on morbidity and mortality: The ILERVAS project. *Nefrologia*, 2016; 36: 389-396
- 11) Kizer JR, Benkeser D, Arnold AM, Ix JH, Mukamal KJ,

- Djousse L, Tracy RP, Siscovick DS, Psaty BM, Ziemann SJ: Advanced glycation/glycoxidation endproduct carboxymethyl-lysine and incidence of coronary heart disease and stroke in older adults. *Atherosclerosis*, 2014; 235: 116-121
- 12) Hanssen NM, Engelen L, Ferreira I, Scheijen JL, Huijberts MS, van Greevenbroek MM, van der Kallen CJ, Dekker JM, Nijpels G, Stehouwer CD, Schalkwijk CG: Plasma levels of advanced glycation endproducts N ϵ -(carboxymethyl)lysine, N ϵ -(carboxyethyl)lysine, and pentosidine are not independently associated with cardiovascular disease in individuals with or without type 2 diabetes: the Hoorn and CODAM studies. *J Clin Endocrinol Metab*, 2013; 98: E1369-E1373
- 13) Busch M, Franke S, Muller A, Wolf M, Gerth J, Ott U, Niwa T, Stein G: Potential cardiovascular risk factors in chronic kidney disease: Ages, total homocysteine and metabolites, and the c-reactive protein. *Kidney Int*, 2004; 66: 338-347
- 14) Bolívar B, Fina Avilés F, Morros R, Garcia-Gil Mdel M, Hermosilla E, Ramos R, Rosell M, Rodríguez J, Medina M, Calero S, Prieto-Alhambra D; Grupo SIDIA. [SIDIA database: electronic clinical records in primary care as a source of information for epidemiologic research]. *Med Clin (Barc)*, 2012; 138: 617-621
- 15) Diabetes mellitus: a major risk factor for cardiovascular disease. A joint editorial statement by the American Diabetes Association; The National Heart, Lung, and Blood Institute; The Juvenile Diabetes Foundation International; The National Institute of Diabetes and Digestive and Kidney Diseases; and The American Heart Association. *Circulation*, 1999; 100: 1132-1133
- 16) Sabetai MM, Tegos TJ, Nicolaides AN, Dhanjil S, Pare GJ, Stevens JM: Reproducibility of computer-quantified carotid plaque echogenicity: can we overcome the subjectivity? *Stroke*, 2000; 31: 2189-2196
- 17) Touboul PJ, Hennerici G, Meairs S, Adams H, Amarenco P, Desvarieux M, Ebrahim S, Fatar M, Hernandez Hernandez R, Kownator S, Prati P, Rundek T, Taylor A, Bornstein N, Csiba L, Vicaut E, Woo KS, Zannad F; Advisory Board of the 3rd Watching the Risk Symposium 2004, 13th European Stroke Conference: Mannheim intima-media thickness consensus. *Cerebrovasc Dis*, 2004; 18: 346-349
- 18) Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, Najjar SS, Rembold CM, Post WS; American Society of Echocardiography Carotid Intima-Media Thickness Task Force: Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American society of echocardiography carotid intima-media thickness task force endorsed by the society for vascular medicine. *J Am Soc Echocardiogr*, 2008; 21: 93-111
- 19) Tiozzo E, Gardener H, Hudson BI, Dong C, Della-Morte D, Crisby M, Goldberg RB, Elkind MS, Cheung YK, Wright CB, Sacco RL, Rundek T: High-density lipoprotein subfractions and carotid plaque: The Northern Manhattan Study. *Atherosclerosis*, 2014; 237: 163-168
- 20) Rundek T, Arif H, Boden-Albala B, Elkind MS, Paik MC, Sacco RL: Carotid plaque, a subclinical precursor of vascular events: the Northern Manhattan Study. *Neurology*, 2008; 70: 1200-1207
- 21) Meerwaldt R, Graaff R, Oomen PHN, Links TP, Jager JJ, Alderson NL, Thorpe SR, Baynes JW, Gans ROB, Smit AJ: Simple non-invasive assessment of advanced glycation endproduct accumulation. *Diabetologia*, 2004; 47: 1324-1330
- 22) Fernández-Friera L, Peñalvo JL, Fernández-Ortiz A, Ibañez B, López-Melgar B, Laclaustra M, Oliva B, Moco-roa A, Mendiguren J, Martínez de Vega V, García L, Molina J, Sánchez-González J, Guzmán G, Alonso-Farto JC, Gual-lar E, Civeira F, Sillesen H, Pocock S, Ordovás JM, Sanz G, Jiménez-Borreguero LJ, Fuster V: Prevalence, vascular distribution, and multiterritorial extent of subclinical atherosclerosis in a middle-aged cohort: the PESA (Progression of Early Subclinical Atherosclerosis) study. *Circulation*, 2015; 131: 2104-2113
- 23) Belcaro G, Nicolaides AN, Ramaswami G, Cesarone MR, De Sanctis M, Incandela L, Ferrari P, Geroulakos G, Barsotti A, Griffin M, Dhanjil S, Sabetai M, Bucci M, Martines G: Carotid and femoral ultrasound morphology screening and cardiovascular events in low risk subjects: a 10-year follow-up study (the CAFES-CAVE study). *Atherosclerosis*, 2001; 156: 379-387
- 24) Baber U, Mehran R, Sartori S, Schoos MM, Sillesen H, Muntendam P, Garcia MJ, Gregson J, Pocock S, Falk E, Fuster V: Prevalence, impact and predictive value of detecting subclinical coronary and carotid atherosclerosis in asymptomatic adults: the BioImage Study. *J Am Coll Cardiol*, 2015; 65: 1065-1074
- 25) Laclaustra M, Casasnovas JA, Fernández-Ortiz A, Fuster V, León-Latre M, Jiménez-Borreguero LJ, Pocovi M, Hurtado-Roca Y, Ordovás JM, Jarauta E, Gual-lar E, Ibañez B, Civeira F: Femoral and carotid subclinical atherosclerosis association with risk factors and coronary calcium: The AWHs Study. *J Am Coll Cardiol*, 2016; 67: 1263-1274
- 26) Mitchell JD, Paisley R, Moon P, Novak E, Villines TC: Coronary artery calcium and long-term risk of death, myocardial infarction, and stroke: The Walter Reed Cohort Study. *JACC Cardiovasc Imaging*, 2017; S1936-878X: 30891-30894
- 27) Janda K, Krzanowski M, Gajda M, Dumnicka P, Jasek E, Fedak D, Pietrzycka A, Kuźniewski M, Litwin JA, Sułowicz W: Vascular effects of advanced glycation end-products: content of immunohistochemically detected AGEs in radial artery samples as a predictor for arterial calcification and cardiovascular risk in asymptomatic patients with chronic kidney disease. *Dis Markers*, 2015; 2015: 153978
- 28) van Eupen MG, Schram MT, van Sloten TT, Scheijen J, Sep SJ, van der Kallen CJ, Dagnelie PC, Koster A, Schaper N, Henry RM, Kroon AA, Smit AJ, Stehouwer CD, Schalkwijk CG: Skin autofluorescence and pentosidine are associated with aortic stiffening: The Maastricht study. *Hypertension*, 2016; 68: 956-963
- 29) Saremi A, Howell S, Schwenke DC, Bahn G, Beisswenger PJ, Reaven PD.; VADT Investigators: Advanced Glycation End Products, Oxidation Products, and the Extent of Atherosclerosis During the VA Diabetes Trial and Follow-up Study. *Diabetes Care*, 2017; 40: 591-598
- 30) Xu L, Wang YR, Li PC, Feng B: Advanced glycation end

- products increase lipids accumulation in macrophages through upregulation of receptor of advanced glycation end products: increasing uptake, esterification and decreasing efflux of cholesterol. *Lipids Health Dis*, 2016; 15: 161
- 31) Ninomiya H, Katakami N, Sato I, Osawa S, Yamamoto Y, Takahara M, Kawamori D, Matsuoka TA, Shimomura I: Association between subclinical atherosclerosis markers and the level of accumulated advanced glycation end-products in the skin of patients with diabetes. *J Atheroscler Thromb*, 2018; 25: 1274-1284
 - 32) Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, Brett J, Cao R, Kuwabara K, Costache G: Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci USA*, 1994; 91: 8807-8811
 - 33) Basta G: Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications. *Atherosclerosis*, 2008; 196: 9-21
 - 34) van Waateringe RP, Slagter SN, van Beek AP, van der Klauw MM, van Vliet-Ostaptchouk JV, Graaff R, Paterson AD, Lutgers HL, Wolffenbuttel BHR: Skin autofluorescence, a non-invasive biomarker for advanced glycation end products, is associated with the metabolic syndrome and its individual components. *Diabetol Metab Syndr*, 2017; 9: 42
 - 35) Mulder DJ, van Haelst PL, Gross S, de Leeuw K, Bijzet J, Graaff R, Gans RO, Zijlstra F, Smit AJ: 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. *Atherosclerosis*, 2008; 197: 217-223
 - 36) de Vos LC, Noordzij MJ, Mulder DJ, Smit AJ, Lutgers HL, Dullaart RP, Kamphuisen PW, Zeebregts CJ, Lefrandt JD: Skin autofluorescence as a measure of advanced glycation end products deposition is elevated in peripheral artery disease. *Arterioscler Thromb Vasc Biol*, 2013; 33: 131-138
 - 37) Hangai M, Takebe N, Honma H, Sasaki A, Chida A, Nakano R, Togashi H, Nakagawa R, Oda T, Matsui M, Yashiro S, Nagasawa K, Kajiwarra T, Takahashi K, Takahashi Y, Satoh J, Ishigaki Y: Association of advanced glycation end products with coronary artery calcification in Japanese subjects with Type 2 Diabetes as assessed by skin autofluorescence. *J Atheroscler Thromb*, 2016; 23: 1178-1187
 - 38) Meerwaldt R, Lutgers HL, Links TP, raaff R, Baynes JW, Gans RO, Smit AJ: Skin autofluorescence is a strong predictor of cardiac mortality in diabetes. *Diabetes Care*, 2007; 30: 107-112
 - 39) Kimura H, Tanaka K, Kanno M, Watanabe K, Hayashi Y, Asahi K, Suzuki H, Sato K, Sakaue M, Terawaki H, Nakayama M, Miyata T, Watanabe T: Skin autofluorescence predicts cardiovascular mortality in patients on chronic hemodialysis. *Ther Apher Dial*, 2014; 18: 461-467
 - 40) Velayoudom-Cephise FL, Rajaobelina K, Helmer C, Nov S, Pupier E, Blanco L, Hugo M, Farges B, Astrugue C, Gin H, Rigalleau V: Skin autofluorescence predicts cardio-renal outcome in type 1 diabetes: a longitudinal study. *Cardiovasc Diabetol*, 2016; 15: 127
 - 41) Sánchez E, Betriu À, Arroyo D, López C, Hernández M, Rius F, Fernández E, Lecube A: Skin Autofluorescence and Subclinical Atherosclerosis in Mild to Moderate Chronic Kidney Disease: A Case-Control Study. *PLoS One*, 2017; 12: e0170778
 - 42) Osawa S, Katakami N, Kuroda A, Takahara M, Sakamoto F, Kawamori D, Matsuoka T, Matsuhisa M, Shimomura I: Skin autofluorescence is associated with early stage atherosclerosis in patients with Type 1 Diabetes. *J Atheroscler Thromb*, 2017; 24: 312-326
 - 43) Barnett PA, Spence JD, Manuck SB, Jennings JR: Psychological stress and the progression of carotid artery disease. *J Hypertens*, 1997; 15: 49-55
 - 44) Semba RD, Bandinelli S, Sun K, Guralnik JM, Ferrucci L: Plasma carboxymethyl-lysine, an advanced glycation end product, and all-cause and cardiovascular disease mortality in older community-dwelling adults. *J Am Geriatr*, 2009; 57: 1874-1880
 - 45) Semba RD, Ferrucci L, Sun K, Beck J, Dalal M, Varadhan R, Walston J, Guralnik JM, Fried LP: Advanced glycation end products and their circulating receptors predict cardiovascular disease mortality in older community-dwelling women. *Aging Clin Exp Res*, 2009; 21: 182-190
 - 46) Kilhovd BK, Juutilainen A, Lehto S, et al.: Rönnekaa T, Torjesen PA, Hanssen KE, Laakso M serum levels of advanced glycation endproducts predict total, cardiovascular and coronary mortality in women with type 2 diabetes: A population-based 18 year follow-up study. *Diabetologia*, 2007; 50: 1409-1417
 - 47) Nin JW, Jorsal A, Ferreira I, Schalkwijk CG, Prins MH, Parving HH, Tarnow L, Rossing P, Stehouwer CD: Higher plasma levels of advanced glycation end products are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: A 12-year follow-up study. *Diabetes Care*, 2011; 34: 442-447
 - 48) van Waateringe RP, Slagter SN, van der Klauw MM, van Vliet-Ostaptchouk JV, Graaff R, Paterson AD, Lutgers HL, Wolffenbuttel BH: Lifestyle and clinical determinants of skin autofluorescence in a population-based cohort study. *Eur J Clin Invest*, 2016; 46: 481-490