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Skin Autofluorescence and Subclinical Atherosclerosis in Mild to Moderate Chronic Kidney Disease: A Case-Control Study

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Abstract

Advanced glycation end-products (AGEs) are increased and predict mortality in patients with chronic kidney disease (CKD) who are undergoing hemodialysis, irrespective of the presence of type 2 diabetes. However, little information exits about the relationship between AGEs and subclinical atherosclerosis at the early stages of CKD. A case-control study was performed including 87 patients with mild-to-moderate stages of CKD (glomerular filtration rate from 89 to 30 ml/min/per 1.73m^2) and 87 non-diabetic non-CKD subjects matched by age, gender, body mass index, and waist circumference. Skin autofluorescence (AF), a non-invasive assessment of AGEs, was measured. The presence of atheromatous disease in carotid and femoral arteries was evaluated using vascular ultrasound, and vascular age and SCORE risk were estimated. Patients with mild-to-moderate stages of CKD showed an increase in skin AF compared with control subjects (2.5±0.6 *vs*. 2.2±0.4 AU, p<0.001). A skin AF value >2.0 AU was accompanied by a 3-fold increased risk of detecting the presence of an atheromathous plaque (OR 3.0, 95% CI 1.4–6.5, p = 0.006). When vascular age was assessed through skin AF, subjects with CKD were almost 12 years older than control subjects (70.3±25.5 *vs*. 58.5±20.2 years, p = 0.001). Skin AF was negatively correlated with glomerular filtration rate (r = -0.354, p<0.001) and LDL-cholesterol (r = -0.269, p = 0.001), and positively correlated with age (r = 0.472, p<0.001), pulse pressure (r = 0.238, p = 0.002), and SCORE risk (r = 0.451, p<0.001). A stepwise multivariate regression analysis showed that age and glomerular filtration rate independently predicted skin AF (R^2 = 0.289, p<0.001). Skin AF is elevated in patients with mild-to-moderate CKD compared with control subjects. This finding may be independently associated with the glomerular filtration rate and the presence of subclinical atheromatous disease. Therefore, the use of skin AF may help to accurately evaluate the real cardiovascular risk at the early stages of CKD.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Advanced glycation end products (AGEs) characterize a heterogeneous group of compounds formed by the non-enzymatic glycation of proteins after exposure to aldose sugars [1]. These reactions progress in normal aging, and are accelerated under chronic hyperglycemia [2, 3]. In this way, the concentration of AGEs is associated with a higher incidence and faster progression of chronic type 2 diabetes (T2D) microangiopathy, and it is also an independent predictor of mortality in this population [4, 5]. In addition, other conditions like chronic inflammation, oxidative stress, and tobacco smoke can lead to increased AGES formation [1, 6, 7].

The gold standard skin biopsy measurement of AGEs agglomeration may be substituted by a non-invasive device based on skin autofluorescence (AF) [8]. Skin AF has been previously validated in clinical settings, and its clinical value has been established in large studies including individuals with a high risk of atherosclerosis, as T2D and chronic kidney disease (CKD) [9–12]. AGEs promote the development and evolution of atherosclerosis through direct and receptor pathways [13].

The progressive loss of glomerular filtration rate (GFR) is associated with systemic inflammation, as well as with an imbalance between oxygen reactive species production and antioxidant defenses [14, 15]. Increased circulating levels of AGEs are found in patients with CKD undergoing hemodialysis regardless, of the presence of T2D [4, 16]. Some additional factors have been associated with AGEs accumulation in renal failure because of decreased glomerular filtration, intraperitoneal formation during the time course of peritoneal dialysis, or dietary intake [17–20]. Therefore, the high body burden of AGEs in subjects with CKD may play a role in the pathogenesis of vascular complications associated with hemodialysis [21]. However, there is little information about the relationship between AGEs and subclinical atherosclerosis at earlier stages of CKD.

To shed light on this issue, we performed a case-control study of tissue accumulation of AGEs according to the presence of mild to moderate CKD. For this purpose, we selected subjects without T2D and no previous cardiovascular events. The AGEs were measured via skin AF. We also aimed to assess the relationship between AGEs accumulation and subclinical atheromatosis, by evaluating vascular ultrasound data.

Material and Methods

Ethics statement

Informed written consent was obtained from all participants, and the protocol was approved by the Arnau de Vilanova University Hospital ethics committee.

Design of the study and description of the study population

We assessed the effect of mild to moderate CKD on tissue accumulation of AGEs following the *Strengthening the Reporting of Observational Studies in Epidemiology* guidelines for reporting case-control studies [22].

A total of 128 patients attending the outpatient Nephrology Clinic were examined to determine eligibility at the time of a regular visit between December 2014 and October 2015. The inclusion criteria were age older than 18 years, Caucasian origin, and GFR categories G2 (mildly decreased; 60–89 ml/min/per 1.73m^2), G3a (mildly to moderately decreased; 45–59 ml/min/per 1.73m^2), or G3b (moderately to severely decreased; 30–44 ml/min/per 1.73m^2) according the standards established by the *Kidney Disease*: *Improving Global Outcomes* [23]. Therefore, all patients with GFR category G2 also present moderately or increased albuminuria

($\geq$ 30 mg/g or $\geq$ 3 mg/mmol). The GFR was estimated following the CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) equation [24].

Using the standard deviation of serum AGEs detected in a previous study, we determined that the minimum sample required was 51 subjects [2]. Forty-one patients were excluded: T2D (n = 10), prior cardiovascular event (n = 8), GFR lower than 30 ml/min/per 1.73m^2 (n = 6), non-Caucasian races (n = 4), chronic treatment with steroids (n = 3), active malignancy (n = 3), type 1 diabetes (n = 3), and age older than 80 years (n = 1). Moreover, 3 patients were excluded for their brown skin (Fitzpatrick type IV skin) because the excessive light absorption produced by this type of skin precludes reliable measurements of skin AF. No pregnant women were evaluated.

We aimed to select one control for every case. Subsequently, 87 subjects without kidney disease (GFR categories G1 and G2 without albuminuria) from the same Department served as the control group. Controls were closely matched to cases by, gender, BMI, waist circumference, and smoking status. As a linear relation between skin AF and subject age has been previously described, both groups were also matched by chronological age [2].

Measurement of AGEs accumulation and determination of vascular age

Skin AF was measured using the AGE Reader™ device (DiagnOptics, Groningen, The Netherlands), a fully automated noninvasive tool that measures AGE deposition using an Ultraviolet-A spectrum. The skin AF is determined from the ratio between the emission fluorescence in the wavelength range between 420–600 nm, and the reflected excitation light with a wavelength between 300–420 nm, which was measured using a spectrometer and software. The measurement time is about one minute, and the mean value of three readings was recorded in all subjects. In addition, vascular age was calculated using skin AF value by the formula previously validated by Koetsier [vascular age = (skin AF– 0.83) / 0.024)] [2].

Vascular ultrasound study and SCORE risk estimation

The ultrasound assessment of carotid and femoral arteries followed a predetermined protocol as defined in the NEFRONA study [25]. Briefly, B-mode and color-Doppler ultrasound imaging was performed using a Vivid-i BT09device (General Electrics Healthcare, Waukesha, WI) equipped with 6–13 MHz broadband linear array transducer and Doppler examinations in transverse and longitudinal planes. The presence of atheromatous plaque in the following territories was evaluated on the left and right sides: internal, bulb and common carotid arteries, and deep and superficial femoral arteries. Plaques were defined as focal intrusions into the lumen $\geq$1.5 mm thick, as recommended by *American Society of Echocardiography* [26]. Simultaneously, the ankle-brachial index (ABI) was assessed: a pathologic ABI was defined as a value $\leq$0.9 or $\geq$1.4, and the modified method by Schröeder was preferred [27]. Participants were classified by grades of atheromatous disease in 4 stages according ultrasound study and the ABI: **(i)** no atherosclerosis (ABI >0.9); **(ii)** mild atherosclerosis (ABI between 0.7–0.9); **(iii)** moderate atherosclerosis (carotid plaque with stenosis <50%); and **(iv)** severe atherosclerosis (ABI <0.7 or carotid plaque with stenosis $\geq$50%) [25]. To better analyze our results, patients were grouped according to the severity of atheromatous disease: Group I (patients without and with mild atherosclerosis, in which the absence of plaques is mandatory) and Group II (patients with moderate and severe atherosclerosis, in which presence of plaques is mandatory).

The SCORE (Systematic COronary Risk Evaluation) risk system is based on age, gender, country of origin, systolic blood pressure, smoking status, and either total cholesterol or total cholesterol/high-density lipoprotein cholesterol ratio. It was used to estimate the 10-year risk of mortality from cardiovascular disease [28].

Statistical analysis

Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Data were expressed either as the mean ± SD or median (total range). Comparisons between groups were performed using the Student's *t* test or the Mann-Whitney U test for continuous variables, and the χ^2 test or the Fisher test were used for categorical variables.

The relationship between the continuous variables was examined with Pearson's linear correlation test or the Spearman correlation coefficient. A stepwise multivariate regression analysis was used to explore the variables independently related to skin AF. The independent variables included age, gender, pulse pressure, LDL cholesterol, GFR, glycosylated haemoglobin, and SCORE risk. Significance was considered with a two-sided p value <0.05. Statistical analyses were performed using SSPS statistical package (SPSS, Chicago, IL, USA) version 20.

Results

The main clinical characteristics and metabolic data of the study population according to the presence of CKD are showed in Table 1. Patients with mild to moderate decrease in GFR showed significantly higher levels of skin AF versus non-CKD subjects (2.5 ± 0.6 *vs*. 2.2 ± 0.4

Table 1. Main clinical characteristics and metabolic data of the study population according to the presence of chronic kidney disease.

	Mild to moderate CKD	*Non CKD*	*Mean difference (95% CI)*	*p*
N	87	87	-	-
Women, n (%)	33 (37.9)	33 (37.9)	-	1.000
Age (yrs)	58.1 ± 10.6	56.5 ± 8.8	-1.5 (-4.4 to 1.4)	0.307
BMI (Kg/m^2)	28.8 ± 5.8	28.9 ± 4.8	0.8 (-1.5 to 1.6)	0.918
Waist circumference (cm)	100.4 ± 15.1	100.7 ± 12.7	0.2 (-3.9 to 4.5)	0.893
Non-smoker, n (%)	56 (49.5)	49 (41.4)	-	0.286
Systolic Blood Pressure (mm Hg)	131.1 ± 16.8	128.0 ± 18.0	-3.0 (-8.2 to 2.1)	0.246
Diastolic Blood Pressure (mm Hg)	77.6 ± 10.0	75.7 ± 11.7	-1.9 (-5.1 to 1.3)	0.253
Pulse Pressure (mm Hg)	53.5 ± 14.3	52.3 ± 14.3	-1.1 (-5.4 to 3.1)	0.591
Fasting plasma glucose (mmol/l)	5.3 ± 0.5	5.3 ± 0.8	0.0 (-3.1 to 4.7)	0.685
HbA1c (%)	5.4 ± 0.3	5.4 ± 0.4	0.0 (-0.1 to 0.1)	0.933
Serum Creatinine (mg/dL)	1.32 ± 0.6	0.81 ± 0.1	-0.5 (-0.6 to -0.3)	<0.001
GFR (mL/min per 1.73m^2)	60.8 ± 18.3	90.0 ± 9.3	29.1 (24.7 to 33.5)	<0.001
ACR (mg/g)	108.2 ± 191.0	5.3 ± 5.3	-102.9 (-150.7 to -55.1)	<0.001
Total cholesterol (mg/dL)	164.5 ± 35.6	194.9 ± 41.9	30.4 (-18.7 to 42.1)	<0.001
HDL-cholesterol (mg/dL)	50.7 ± 12.6	53.2 ± 12.2	2.42 (-1.4 to 6.2)	0.218
LDL-cholesterol (mg/dL)	91.4 ± 27.9	117.4 ± 37.2	26.0 (15.8 to 36.3)	<0.001
Triglycerides (mg/dL)	133.5 (42.0 to 780.0)	140.0 (52.0 to 632)	6.4 (-21.6 to 34.6)	0.650
SCORE risk (%)	2.3 ± 2.6	1.7 ± 2.2	-0.6 (-1.3 to 0.0)	0.079
Atheromatous plaque, n (%)	68 (78.1)	72 (82.7)	-	0.444
Causes of chronic kidney disease				
High blood pressure	36 (41.4)	-	-	-
Polycystic kidney disease	16 (18.4)	-	-	-
Glomerulonephritis	25 (28.7)	-	-	-
Tubulointerstitial nephritis	10 (11.5)	-	-	-

Data are means ± SD, n (percentage) or median (total range). CKD: chronic kidney disease; BMI: body mass index; HbA1c: glycosylated haemoglobin; GFR: glomerular filtration rate estimated according the CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) equation; ACR: albumin to creatinine ratio; SCORE: Systematic Coronary Risk Evaluation.

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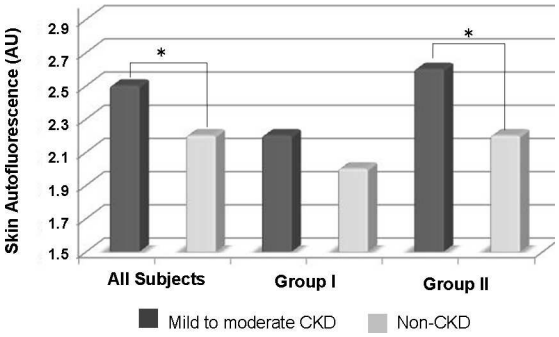

Fig 1. Skin autofluorescence according to the presence of atheromatous plaque between controls and patients with mild to moderate stages of CKD. CKD: chronic kidney disease; AU: arbitrary units; *: p<0.001; Group I: patients without and with mild atherosclerosis, in which the absence of plaques is mandatory; Group II: patients with moderate and severe atherosclerosis, in which presence of plaques is mandatory.

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arbitrary units (AU), p<0.001). When the subjects with and without atheromatous plaque (group I vs. group II) were analyzed separately, differences in skin AF values persisted only in the second group (Group II: 2.6 ± 0.5 *vs*. 2.2 ± 0.5 AU, p<0.001), and disappeared among subjects with no detectable plaque (Group I: 2.2 ± 0.7 *vs*. 2.0 ± 0.3 AU, p = 0.464) (Fig 1). When the entire population was evaluated, subjects with a skin AF value higher > 2.0 AU showed a 3-fold increased risk of an atheromathous plaque (OR 3.0, 95% CI 1.4–6.5, p = 0.006).

As shown in previous studies, a strong positive correlation was observed between skin AF and age (r = 0.472, p<0.001), without differences between genders. While both groups were closely matched for age, when vascular age was assessed, subjects with mild to moderate CKD appeared to be almost twelve years older than control subjects (70.3 ± 25.5 *vs*. 58.5 ± 20.2 years, p = 0.001).

In the entire population, skin AF correlated negatively with GFR (r = -0.354, p<0.001), and LDL-cholesterol (r = -0.269, p = 0.001), and correlated positively with age (r = 0.472, p<0.001), pulse pressure (r = 0.238, p = 0.002), and SCORE risk (r = 0.451, p<0.001). (Fig 2). The same linear correlations were observed when only patients with CKD were evaluated, but disappeared in the control group. An intriguing negative correlation was also stablished between AGEs and LDL cholesterol (Table 2).

Finally, a stepwise multivariate regression analysis showed that the age and GFR (but not pulse pressure, glycosylated hemoglobin, LDL-cholesterol nor SCORE risk) were independently associated with forearm skin AF (R^2 = 0.289, p<0.001) (Table 3).

Discussion

To the best of our knowledge, this is the first study to show that subjects with early stages of CKD significantly increase skin AF values. In addition, a close relationship exists between skin AF and asymptomatic atheromatous disease in this population. Furthermore, skin AF appears to be negatively correlated with GFR, suggesting that renal dysfunction is a key factor to increase AGEs deposition in subcutaneous tissue.

The current study agrees with recent findings in subjects with an estimated GFR>60 ml/min/per 1.73m^2, in whom skin AF was significantly higher in those with peripheral artery disease versus the subclinical atherosclerosis group. This contributes to vascular damage in addition to classical mechanisms [29]. The biological effects of AGEs through its ligation to their receptors located in large blood vessels accelerate plaque formation [13]. In patients with end-stage renal disease, immunostained pentosidine -a major glycoxidation product- was observed

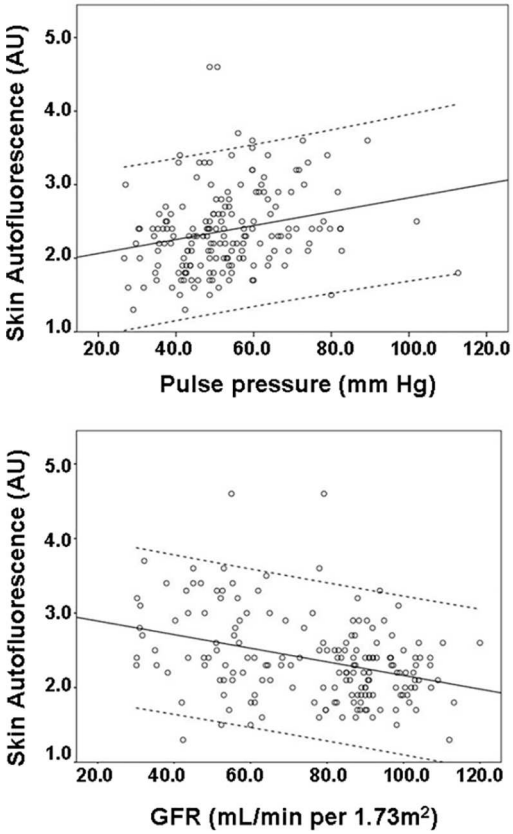

Fig 2. Scatter plot showing linear correlation between skin autofluorescence and: pulse pressure, and glomerular filtration rate. AU: arbitrary units; GFR: glomerular filtration rate estimated according the CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) equation.

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along the elastin fibers in aortic media. This was associated with medial calcification [30]. These data also shown that deposition of AGEs accompanies subclinical atherosclerosis beyond the presence of T2D [31].

Table 2. Correlations of skin autofluorescence with clinic and metabolic variables.

	All subjects		*Mild to moderate CKD*		*Non CKD*	
	r	***p***	***r***	***p***	***r***	***p***
Age (yrs)	0.472	<0.001	0.586	<0.001	0.291	0.006
BMI (kg/m^2)	0.040	0.600	0.088	0.417	-0.25	0.819
PP (mmHg)	0.238	0.002	0.380	<0.001	0.055	0.612
FPG (mmol/l)	-0.021	0.789	0.016	0.885	-0.040	0.712
HbA1c (%)	0.054	0.603	0.139	0.307	-0.124	0.453
GFR (mL/min per 1.73m^2)	-0.349	<0.001	-0.315	0.003	-0.110	0.309
LDL-cholesterol (mg/dl)	-0.269	0.001	-0.127	0.255	-0.284	0.011
SCORE risk (%)	0.451	<0.001	0.541	<0.001	0.314	0.105

CKD: chronic kidney disease; BMI: body mass index; PP: pulse pressure; FPG: fasting plasma glucose; HbA1c: glycosilated hemoglobin; GFR: glomerular filtration rate estimated according the CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) equation; SCORE: Systematic Coronary Risk Evaluation).

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Table 3. Stepwise multivariate regression analysis of variables associated with skin autofluorescence.

		Beta	p
Skin AF	**Age (yrs)**	0.424	<0.001
	GFR (mL/min per 1.73m^2)	-0.275	<0.001
	LDL-cholesterol (mg/dl)	-0.148	0.112
	SCORE risk (%)	0.155	0.171
	PP (mmHg)	0.057	0.598
	HbA1c (%)	0.032	0.719
R^2 = 0.289	*Constant*		0.001

Beta: Standardized regression coefficient; AF: autofluorescence; GFR: glomerular filtration rate estimated according the CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) equation; SCORE (Systematic Coronary Risk Evaluation); PP: pulse pressure; HbA1c: glycosilated haemoglobin.

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CKD substantially increases the risk of cardiovascular disease. Indeed, a large community-based population study including 1,120,295 adults showed an adjusted hazard ratio for cardiovascular events was 1.4 with an estimated GFR of 45 to 59 ml/min/per 1.73m^2. This increased to 2.0 with an estimated GFR of 30 to 44 ml/min/per 1.73m^2 [32]. In addition, AGEs are also known to accumulate in the microvasculature of the kidney and to promote glomerular filtration and proteinuria [33]. In fact, clinical studies in patients with type 1 diabetes revealed a significant increase in the skin concentration of AGEs as urinary albumin increased from normal to microalbuminuria, and macroalbuminuria [34].

The plasma concentration of AGEs in T2D has also identified those normoalbuminuric subjects who will experience a higher increase in the glomerular basement membrane at in about 5-years follow-up period [35]. More recently, Luo et al. have demonstrated that in a non-hyperglycemic milieu, AGEs increase the permeability of the glomerular endothelial cells by a matrix metalloproteinases degradation of tight junction complexes, mainly occluding and claudin-5 proteins [36]. Our results support the close relationship between AGEs accumulation and decreases in GFR because a strong and negative relationship between mild to moderate ranges of GFR and skin AF was observed. Whether the AGEs renal accumulation in humans promotes kidney dysfunction or whether the decrease in GRF triggers AGE accumulation cannot be elucidated from our study. However, when the receptor for AGEs is deleted in a mouse model, there is a 29% increase in GFR accompanied by structural changes such as reduced thickening of glomerular basement membrane and mesangial sclerosis [37].

When assessed using skin AF values, we observed a marked increase in vascular age, which is more than 10 years higher than the chronological age in patients with CKD. Vascular aging occurs along with endothelial dysfunction, vascular remodelling, inflammation, and increased stiffness, all of them previously associated with AGEs [16, 38]. In this way, we observed a 3-fold increased risk of an atheromathous plaque in subjects with a skin AF value higher $>$ 2.0 AU. This data support the idea that AGEs are useful in identifying a subclinical phenotype of early vascular disease in large blood vessels [39]. Therefore, in a CKD population before end-stage disease is established, skin AF may represent a clinically helpful and non-invasive method to screen ~~assess~~ cardiovascular risk.

The relationship between skin AF and other conventional risk factors outside T2D remains controversial. In our study, skin AF positively correlated with SCORE risk when the entire population as well as patients with renal impairment were evaluated. However, the correlation disappeared in the control group. Similarly, skin AF was not related to SCORE risk or its

components in a sub-study of the *Groningen Overweight and Lifestyle* (GOAL) project that included overweight and obese subjects without T2D nor renal disease [40]. These data support the idea that, in the clinical setting the decreased GFR is as a key factor accounting for skin AF when T2D is not present. The inverse association between skin AF and LDL cholesterol detected in our population deserves an additional comment. When the LDL conjugated diene is measured as marker of lipid peroxidative stress, a negative correlation with skin AF has been described in critically ill patients [41]. In addition, serum LDL cholesterol was also negatively correlated with skin AF in a cross-sectional study of 223 individuals visiting the vascular outpatient clinic for primary or secondary prevention [29].

This study has some limitations. As a cross-sectional study, we cannot establish a causal relationship between skin AF and subclinical atheromatosis. However, the problem is clinically relevant since the prevalence of CKD reaches 20.4% among participants from the 2005–2006 *National Health and Nutrition Examination Survey* (NHANES), and help is needed to better identify subjects at risk [42, 43]. Second, we did not compared skin AF with plasma AGEs levels. We assumed that skin AF remains stable for a long time because it is less influenced by factors such as smoking or nutrition. In fact, plasma AGEs measurements were not different when comparing individual with and without cardiovascular disease in participants from two Dutch cohort studies including 1.291 subjects with various degrees of glucose metabolism [44]. Third, skin AF could be unreliable in subjects with dark skin due to excessive light absorption. We tried to solve this limitation selecting only Caucasian subjects and excluding four of them with medium brown skin.

In conclusion, skin AF is elevated in patients with mild to moderate CKD in comparison with control subjects. This finding is related with the presence of subclinical atheromatous disease, and appears to be independently associated with the GFR. Therefore, skin AF is an easy, fast and non-invasive method that may help to accurately evaluate real cardiovascular risk in the early stages of CKD.

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Author Contributions

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Formal analysis: ES AB DA AL.

Funding acquisition: EF AL.

Investigation: ES CL MH FR AL.

Methodology: ES AB EF AL.

Project administration: AB EF AL.

Resources: EF DA AL.

Supervision: AB DA EF AL.

Validation: ES AB.

Visualization: ES AL.

Writing – original draft: ES AB DA AL.

Writing – review & editing: ES AB DA CL MH FR EF AL.

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