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Research article

Is skin autofluorescence (SAF) representative of dermal advanced glycation endproducts (AGEs) in dark skin? A pilot study



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ABSTRACT

Aims: Non-invasively assessed skin autofluorescence (SAF) measures advanced glycation endproducts (AGEs) in the dermis. SAF correlates with dermal AGEs in Caucasians and Asians, but studies in dark-skinned subjects are lacking. In this pilot we aimed to assess whether SAF signal is representative of intrinsic fluorescence (IF) and AGE accumulation in dark skin.

Methods: Skin biopsies were obtained in 12 dark-skinned subjects (6 healthy subjects, median age 22 years; 6 diabetes mellitus (DM) subjects, 65 years). SAF was measured with the AGE Reader, IF using confocal microscopy, and AGE distribution with specific antibodies. CML and MG-H1 were quantified with UPLC-MS/MS and pentosidine with HPLC and fluorescent detection.

Results: SAF correlated with IF from the dermis (405nm, $r = 0.58$, $p < 0.05$), but not with CML ($r = 0.54$, $p = 0.07$). CML correlated with IF from the dermis (405nm, $r = 0.90$, $p < 0.01$). UV reflectance and the coefficient of variation of SAF were negatively correlated ($r = -0.80$, $p < 0.01$). CML and MG-H1 were predominantly present around blood vessels, in collagen and fibroblasts in the dermis.

Conclusion: This proof of concept study is the first to compare non-invasive SAF with AGE levels measured in skin biopsies in dark-skinned subjects. SAF did not correlate with individual AGEs from biopsies, but was associated with IF. However, the intra-individual variance was high, limiting its application in dark-skinned subjects on an individual basis.

1. Introduction

Advanced glycation endproducts (AGEs) accumulate during aging [1] and are acknowledged for their role in the development of several chronic diseases [2, 3]. Their formation is heterogeneous, although primarily being induced by hyperglycemia and oxidative stress [1]. AGEs enhance inflammation by binding to the receptor for AGE (RAGE). Furthermore, AGEs generate cross-links between proteins and accumulate in tissues with slow protein turnover (e.g. collagen) such as skin [4, 5] and vascular tissues [6]. Both pathways are involved in the pathogenesis of age-related chronic diseases.

Skin autofluorescence (SAF) is a non-invasive proxy of dermal accumulation of AGEs [4, 5]. Previous studies have shown in Caucasian [4, 5] and Asian subjects [7] that SAF correlates with several AGEs, including the major AGEs (N^ε-(carboxymethyl)lysine [CML] and pentosidine). Several studies have shown increased SAF levels in type 1 diabetes (T1D) and T2D [3], especially in those with diabetic complications [8, 9], and in renal failure [2, 10]. In addition, SAF is an independent predictor of cardiovascular events in several high-risk groups [11] and predictor of the development of T2D, cardiovascular disease and mortality in the general population [12].

Studies investigating SAF have mainly focused on Caucasians and Asians [13], because of the compromised reliability of the measurement

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in subjects with a dark skin type. A previous study from our research group [14] showed that SAF was generally lower in subjects with dark skin compared to lighter skin. This is thought to be due to increased absorption of excitation and emission light by skin constituents (e.g., melanin), and not because of actual lower AGE levels.

Koetsier *et al.* [15] described an algorithm for the AGE Reader SU, to correct for skin color based on a reflectance level <12%, in order to calculate SAF independent from skin color. Using this algorithm, the corrected SAF values demonstrated similar relations with age as for subjects with a reflectance >12% [15]. Compromised SAF measurements occur predominantly when UV reflectance values fall below approximately 6%, which is usually the case in subjects with high skin pigmentation (Fitzpatrick class V-VI) [16].

SAF has been validated against levels of biochemically assessed AGEs in skin biopsies in Caucasians [4, 5, 17]. In addition, SAF correlated with AGE levels in skin samples of Asian subjects with and without diabetes [7]. However, it is largely unknown what the exact localization is of different AGEs and intrinsic fluorescence in biopsies in dark skin.

The aim of our exploratory pilot study was to assess whether the non-invasively assessed SAF signal in dark-skinned subjects is representative of invasively assessed intrinsic fluorescence and of AGE accumulation in skin biopsies. Additionally, we assessed AGE localization differences between younger healthy and older diabetic dark-skinned subjects. These two small groups were designed expecting a high contrast in AGE accumulation between the groups, as previously seen in Caucasians and Asians.

2. Subjects

We performed a cross-sectional pilot study in 12 subjects with dark skin types (Fitzpatrick IV-VI, increasingly dark). This group consisted of 6 older diabetes mellitus (DM), type 1 and 2, subjects, and 6 healthy young subjects.

Exclusion criteria for all participants were chronic kidney disease class 4 or higher, local skin disease on target skin location, presence of tattoos on target skin location or any contra-indication against interrupting the skin barrier. Clinical data regarding cardiovascular risk profile and medical history were collected. Non-fasting glucose was measured using a blood glucose meter with finger-stick. HbA1c and s-creatinine/estimated glomerular filtration rate (eGFR) were retrieved from recent (<1 month) patient records. The study was approved by the local Medical Ethics Committee of the University Medical Center Groningen (METc 2016/258), complied with the Declaration of Helsinki and all participants gave written informed consent.

3. Materials and methods

3.1. AGE Reader

Non-invasively assessed SAF was obtained with autofluorescence (AGE Reader mu, DiagnOptics Technologies, NL). Excitation light, with a peak wavelength of approximately 375 nm, is sent into the skin. SAF is calculated by the AGE Reader as the ratio between emission light (420–560 nm) emitted by fluorescent molecules in the skin and reflected excitation light (300–420 nm), multiplied by 100 and expressed as arbitrary units (AU) and corrected at low skin reflectance. Reflectance is the calibrated reflectance at 375 nm. SAF is known to correlate with AGE concentrations, both fluorescent and non-fluorescent, in dermal biopsies obtained on the same site [4, 5]. SAF measurements taken over 1 single day have been shown to have an intra-individual Altman error percentage of 5.03% in Caucasian subjects [4, 5].

3.2. Skin biopsies

Skin biopsies and measurements of SAF and skin pigmentation were all performed during one visit, on a standardized location on the volar

side of the forearm, approximately 10 cm below the elbow crease. After subcutaneous local anesthesia with lidocaine solution (which was also confirmed not to be fluorescent), just outside the intended biopsy area, two 3-mm punch skin biopsies were taken and frozen in liquid nitrogen (-196 °C). Afterwards, the biopsies were transferred to a -80 °C freezer.

3.3. Skin photo type assessment by the Mexameter

Skin photo type was quantified as skin pigmentation (Melanin index) and skin redness (Erythema index), based on the concentration of hemoglobin and melanin in the skin using the Mexameter MX 18 (CK electronic, Köln, GER) [18, 19]. Emitted light, in three wavelengths, is reflected by skin and measured by a receiver. Photo type IV is determined by a melanin content of 250–350, photo type V of 350–450 and photo type VI of 450–999. A determination of the photo type by only measuring the melanin is not possible due to overlapping wavelength ranges with redness.

3.4. Immunohistochemistry, intrinsic confocal microscopy fluorescence, UPLC-MS/MS and HPLC analysis

Several measurements were performed on transverse slices of skin biopsies. Immunohistochemical staining was conducted with the anti-AGE antibodies anti-CML (ab30917, Abcam, Cambridge, UK) and anti-N^δ-(5-hydroxy-5-methyl-4-imidazolone-2-yl)-ornithine ([MG-H1], STA-011, cell biolabs, inc., NL) using goat anti-mouse IgG alkaline phosphatase (AP) conjugate ([Go-a-Mo-AP], D0486, Dako, Glostrup, DK) as chromogenic reporter. Histological localization of CML and MG-H1 was evaluated semi-quantitatively by two independent investigators (I.M.A and G.F.H.D). Hematoxylin and eosin (HE) stains were added to be able to identify individual cells.

Invasively assessed intrinsic fluorescence was measured by confocal microscopy using the ZEISS LSM 780 NLO (Zeiss, GER) and quantified by ImageJ. We used single (405nm and 440nm) and 2-photon (750nm, equivalent to 375nm) excitation, which corresponds to the excitation area of the AGE Reader.

In the second skin biopsy, including epidermis and dermis, the concentrations of CML, MG-H1 and pentosidine were assessed by ultra-performance liquid chromatography tandem mass spectrometry measurements (UPLC-MS/MS) and high-performance liquid chromatography (HPLC), respectively [20, 21, 22]. (Supplementary material).

3.5. Statistical analysis

Statistical analysis was limited due to the design of a pilot study, including the small number of subjects. We combined both groups to assess associations between SAF and skin biopsy derived data. When reported, data are shown as median [IQR] or number. The coefficient of variation of SAF was calculated from 3 SAF measurements. Differences in characteristics were assessed using Mann-Whitney U tests, whereas correlations were assessed using Spearman's correlations.

4. Results

4.1. Participant characteristics

The participant characteristics are shown in Table 1 and Supplementary Table 1. Median age of older DM subjects was 65 [53–68] years, of healthy young subjects 22 [20–28] years. The duration of DM was 15 [0–29] years.

SAF was 2.0 [1.6–3.1] AU in older DM subjects versus 1.4 [1.3–1.7] AU in healthy young subjects. Analytically assessed CML, MG-H1 and pentosidine concentrations in older DM subjects (858 nmol/mmol hydroxyproline [778–954]; 1648 [1245–2288]; 13 [11–16]) were higher compared to healthy young subjects (250 nmol/mmol hydroxyproline [203–459]; 1274 [1010–1612]; 3 [3–6]). For all excitation wavelengths

Table 1. Participant characteristics of older diabetic and healthy young subjects. Median [IQR], or n, are given.

	Diabetic subjects	Healthy subjects
N	6	6
Age, y	65 [53–68]	22 [20–28]
Male	5	4
Current smoker	3	1
Body Mass Index, kg/m ²	23 [21–29]	23 [21–25]
Diabetes mellitus	6	0
Duration of diabetes mellitus, y	15 [0–29]	N/A
HbA1c, mmol/mol	62.5 [47.8–79.0]	N/A
eGFR, ml/(min/1.73m ²)	67.5 [53.5–94.0]	N/A
Not fasting glucose, mmol/l	8.7 [6.4–20.6]	5.9 [5.5–6.2]
Anti-hypertensive drugs	6	N/A
Lipid-lowering drugs	5	N/A
Anticoagulant therapy	4	N/A
Cardiovascular comorbidity	3	N/A

Cardiovascular comorbidity is defined as a history of stroke, cardiovascular diseases and/or use of anticoagulant therapy. HbA1c, hemoglobin A1c; eGFR, estimated glomerular filtration rate. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [40].

the average emission from the dermis in older DM subjects (405nm, 437 [296–453]; 440nm, 486 [366–540]; 750nm, 485 [134–563]) was higher compared to healthy young subjects (405nm, 113 [90–147]; 440nm, 155 [143–201]; 750nm, 173 [119–259]).

The average emission from the epidermis for 2-photon 750 nm was higher in older DM subjects compared to healthy young subjects. The reverse applied for 405 nm and 440 nm excitation (Figure 1).

4.2. Influence of skin pigmentation

The melanin index showed higher skin pigmentation in the older DM subjects (594.5 [431.6–722.1]) versus the healthy young subjects (320.8 [272.2–489.5]) and the UV reflectance showed a somewhat lower reflectance (0.06 [0.05–0.07]) in the older DM subjects versus the healthy young subjects (0.07 [0.06–0.08]). There was no correlation between SAF and melanin index ($r = -0.06$, $p = 0.86$) or UV reflectance ($r = -0.12$, $p = 0.72$). A negative correlation existed between UV reflectance and the coefficient of variation of SAF ($r = -0.80$, $p < 0.01$) (Figure 2).

4.3. Relationship of SAF with intrinsic fluorescence and AGE concentrations

SAF correlated significantly with intrinsic fluorescence from the dermis when exciting with 405 nm ($r = 0.58$, $p < 0.05$) and 440 nm ($r = 0.65$, $p < 0.05$), but not with 750 nm ($r = 0.47$, $p = 0.12$). SAF did not correlate with intrinsic fluorescence from the epidermis (405nm, $r = -0.37$, $p = 0.24$; 440nm, $r = -0.40$, $p = 0.20$; 750nm, $r = -0.06$, $p = 0.85$). Furthermore, SAF showed no significant correlation with CML ($r = 0.54$, $p = 0.07$), pentosidine ($r = 0.46$, $p = 0.13$) and MG-H1 ($r = -0.08$, $p = 0.81$). (Figure 3).

4.4. Relationship of intrinsic fluorescence with AGE concentrations

Intrinsic fluorescence, as measured in the dermis of the skin biopsy, correlated strongly with CML when exciting with 405 nm ($r = 0.90$, $p < 0.01$) and 440 nm ($r = 0.75$, $p < 0.01$), but not with 750 nm ($r = 0.45$, $p = 0.15$). Additionally, intrinsic fluorescence (measured in the dermis) correlated significantly with pentosidine when exciting with 405 nm ($r = 0.79$, $p < 0.01$). A borderline significant correlation was present when exciting with 440 nm ($r = 0.57$, $p = 0.05$) and 750 nm ($r = 0.55$, $p = 0.06$). Intrinsic fluorescence did not correlate with MG-H1. CML also correlated significantly with pentosidine ($r = 0.93$, $p < 0.01$), but not with MG-H1 ($r = 0.41$, $p = 0.19$). Moreover, MG-H1 did not correlate with pentosidine ($r = 0.44$, $p = 0.15$).

4.5. Localization of AGEs by immunohistochemistry

Representative staining of AGEs in older DM subjects is shown in Figure 4a, c, e and g, in healthy young subjects in Figure 4b, d, f and h. CML (Figure 4a) and MG-H1 (Figure 4c) were predominantly prominent around blood vessels, in collagen and fibroblasts in the dermis of older DM subjects. CML (Figure 4b) showed presence in dermal fibroblasts of healthy young subjects, and MG-H1 (Figure 4d) was located around blood vessels and also in dermal fibroblasts. In addition, MG-H1 was located in the epidermis in both groups. Moreover, we found positive staining of endothelial cells in the negative controls (i.e., with omission of the primary antibody).

Comparison between both groups demonstrated that CML was more often present around blood vessels and in collagen in the dermis of older DM subjects. MG-H1 staining of collagen was also more often present in older DM subjects. Furthermore, MG-H1 staining of fibroblasts was more often present in healthy young subjects.

Results of immunohistochemistry with anti-pentosidine were not mentioned in the current manuscript, due to the inability to stain pentosidine with the used antibody (orb27502, Biorbyt Ltd., Cambridge, UK).

5. Discussion

To our knowledge, this proof of concept study is the first to investigate advanced glycation endproducts (AGEs) in skin tissue from dark-skinned subjects. The approach was novel compared to other studies in dark-skinned subjects because of using both invasive and non-invasive techniques. In previous studies concerning AGEs in skin tissue, mainly performed in Caucasian subjects, dark-skinned subjects were underrepresented. This study is the first study to have taken skin biopsies in dark-skinned subjects comparing the non-invasive SAF method with AGE levels measured by well-established biochemical techniques and intrinsic fluorescence. Our main finding is that SAF levels are associated with intrinsic fluorescence in the dermis of dark-skinned subjects, but not with CML, pentosidine and MG-H1. Our results extend previous studies demonstrating that SAF is correlated with intrinsic fluorescence in skin biopsies of Caucasians and Asians. Although, the correlation with individual AGEs is limited due to a high intra-individual variance of the SAF measurement in dark skin. Therefore, the SAF measurement is not reliable on an individual level in dark-skinned subjects, and attempts to improve the non-invasive SAF measurement should be encouraged. Our results might extend the research on SAF as a non-invasive representation of AGEs located in dark skin.

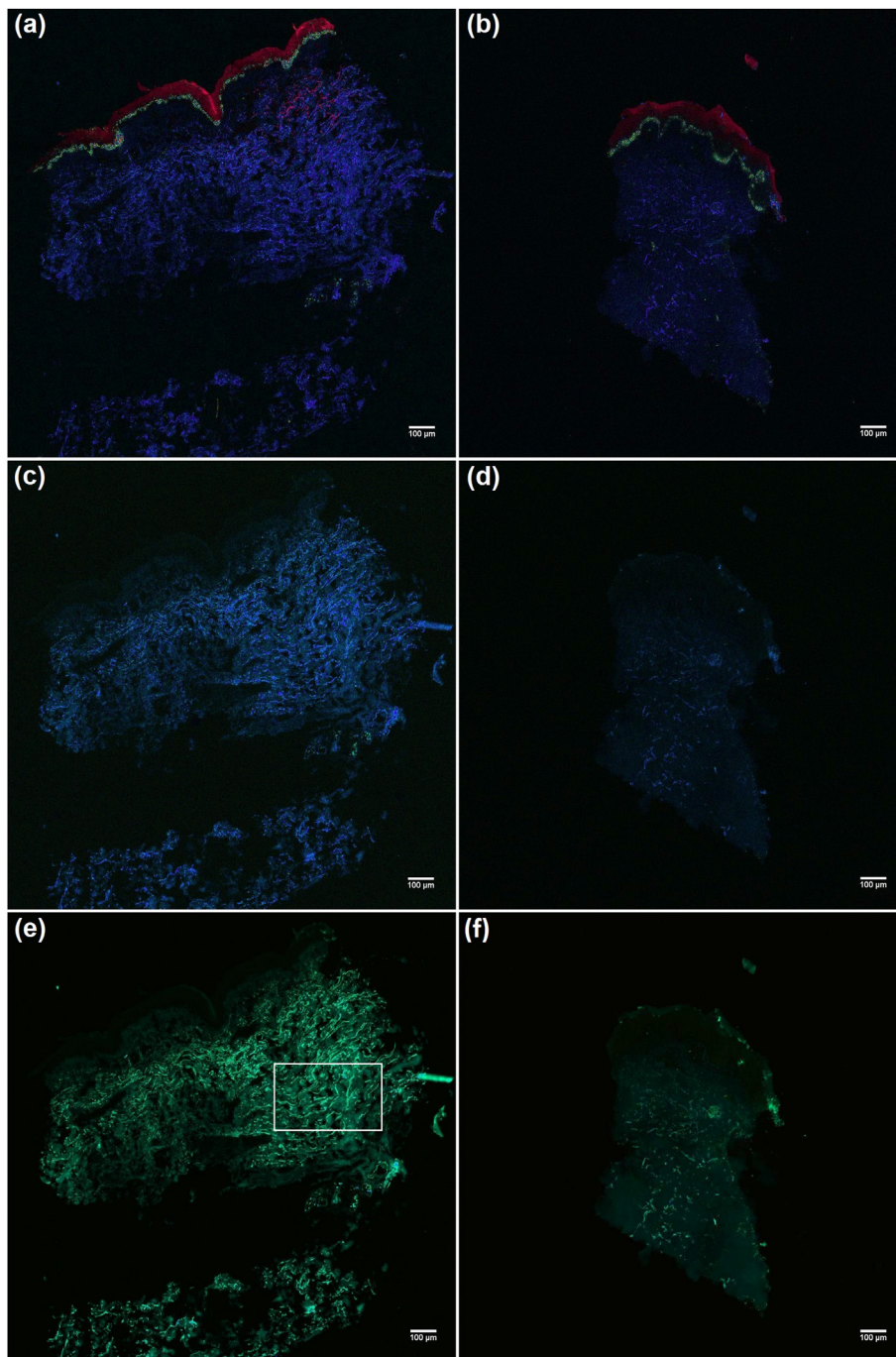


Figure 1. Intrinsic confocal microscopy fluorescence using excitation with 750 nm (2-photon) (a), 405 nm (single photon) (c) and 440 nm (single photon) (e) in representative images of skin biopsies of older diabetic and healthy young subjects (b, d, f). A power of 80% and a gain of 908 was used for 750 nm excitation. A power of 0,2% and a gain of 755 was used for 405 nm excitation and a power of 20% and a gain of 578 was used for 440 nm excitation. Magnification: x20. The square represents collagen. Scale bar = 100 μm .

Since the SAF measurement is validated on the volar forearm, the measurement was performed at the same location as in previous studies, allowing comparison. Less pigmented areas, like palms or plants, may not be suitable since the epidermal layer is usually considerably thicker, and also more variable between persons and within a person at different time points, limiting reliable use of SAF for assessing AGE levels at these skin sites.

However, a recent study by Kim *et al.* [23] found that, using a novel SAF instrument, SAF was significantly higher at palmoplantar sites in Asian subjects with T2D complications, as compared to those without. No differences were observed between these groups in SAF of non-palmoplantar sites. This should open the field for further studies in less pigmented areas.

Although our study shows novel results regarding AGEs in dark skin, consisting of biopsy samples and non-invasive SAF measurements, the issue of interpretation of SAF values in subjects with very dark skin (e.g.,

Fitzpatrick V-VI) has not yet been solved [13, 24, 25]. Koetsier *et al.* [15] attempted to compute reliable corrections for melanin content of skin, using an additional white light, but this was only successful in subjects with moderate skin pigmentation. Our findings seem to be in line with the publications concerning measurement of SAF using another commercialized device (SCOUT) with which measurements also are problematic in a subset of subjects with a dark skin type [26].

Future studies should focus at sites with lower pigmentation, such as palms or plants, or at limiting the confounding effect of other chromo- and fluorophores in the skin which may absorb excitation light, when assessing new SAF measurement algorithms. Moreover, the use of polarizers is recommended. When polarizers are crossed, surface reflection will be eliminated, enhancing the measurement of autofluorescence from the dermis [27, 28, 29]. This may further improve the SAF measurement, especially in very dark-skinned subjects.

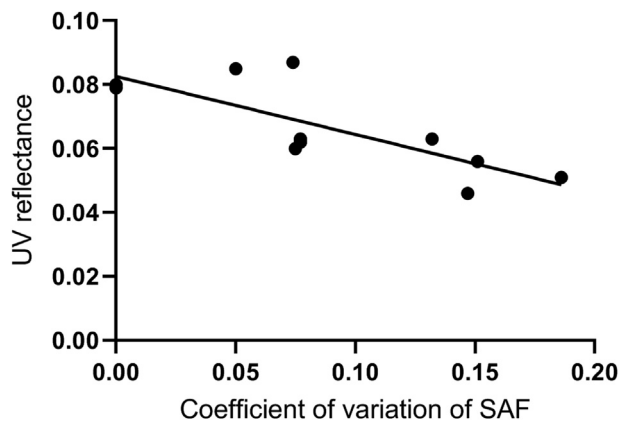


Figure 2. The association between UV reflectance and the coefficient of variation of skin autofluorescence (SAF).

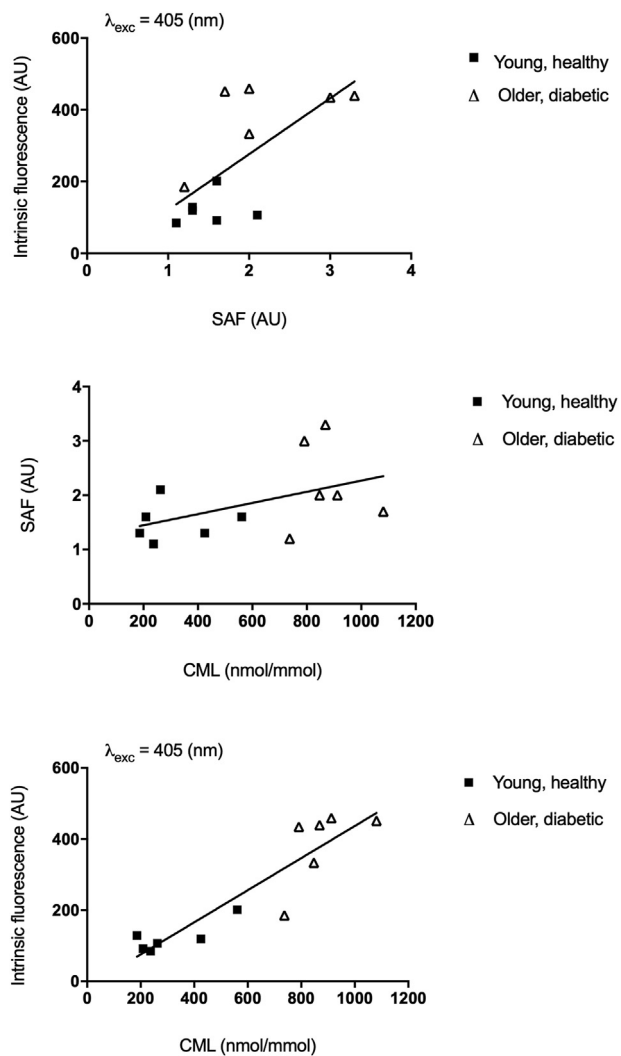


Figure 3. The relationship of skin autofluorescence (SAF) with intrinsic fluorescence and analytically assessed CML.

Previously, our research group compared SAF with biochemically assessed AGEs in Caucasian skin using UPLC-MS/MS and HPLC [4, 5]. Meerwaldt *et al.* [4, 5] reported a strong correlation between SAF and AGEs in skin biopsies from mainly Caucasian diabetic subjects and non-diabetic control subjects. Although the association of SAF with CML, pentosidine, and MG-H1 was not significant, our study did show a

significant association with intrinsic fluorescence in dark-skinned subjects.

In addition, although it was not the objective of our study, we found an increase of biochemically assessed AGEs in skin of older DM subjects compared to healthy younger subjects. The amount of CML, pentosidine and MG-H1 was higher in the older DM subjects. This is in accordance with previous studies investigating AGE levels in Caucasians [4, 5, 17] and Asians [7].

MG-H1 did not correlate with SAF, intrinsic fluorescence, CML and pentosidine. MG-H1 is an AGE derived from the reaction of methylglyoxal with arginine, while CML is derived from glyoxal, and pentosidine from ribose, the last one occurring in the slow Maillard reaction. In the light of these different formation pathways, it is not a surprise that these AGEs may not be related. Also, we found a notable presence of MG-H1 in the epidermis, which may partly explain this finding. Moreover, previous studies in different body compartments (serum and lens proteins) have shown mixed results [30, 31].

Finally, intrinsic fluorescence by confocal microscopy was more prominent in the dermis of older DM subjects, confirming the accumulation of endogenous fluorophores in dark skin, with aging and/or glycaemic stress. A previously conducted study by Tseng *et al.* [32] found that multiphoton autofluorescence (435–700nm) intensity was increased in glycated tissue, including skin, compared to non-glycated tissue. However, previous studies investigating the optical detection of AGEs using confocal microscopy were so far lacking.

The current study was able to localize specific AGEs in different components of dark skin. More particularly, CML and MG-H1 were present around blood vessels, and colocalized with collagen and dermal fibroblasts. This is in accordance with earlier studies that found an increased CML deposition in long-living tissues (e.g., collagen) [33] and fibroblasts in skin with aging [34, 35]. In addition, a previously conducted study by Murata *et al.* [36] showed that CML was accumulated around vessels in human diabetic retinas.

Furthermore, CML staining was more prominent around blood vessels and in collagen, whereas deposition of MG-H1 was more prominent in collagen of older DM subjects, as compared to younger subjects. Our finding is supported by a previous study by Schleicher *et al.* [37], stating that CML was increased in dermal connective tissue of older DM subjects, as compared to younger subjects. Our study is, so far, the first to report MG-H1 deposition in the skin.

The present study has some unexpected findings. MG-H1 staining was more often present in fibroblasts of healthy young subjects, in contrast to older subjects with DM. This is in contrast to our expectations, based on behavior of AGEs during aging. Since aging is associated with a decrease in dermal blood vessels [38] and decreased collagen turnover, due to a decrease in fibroblasts [39], this might explain the increased MG-H1 expression in fibroblasts of healthy young subjects.

In addition, for CML and pentosidine we found a strong correlation with intrinsic fluorescence from the dermis for single photon confocal microscopy. For 2-photon microscopy (750nm) this correlation was less clear, which might be explained by the lower signal-to-noise in the 2-photon images.

The present study has some limitations. First, skin color differed between our two groups, with a higher melanin index in the elderly. Blood testing has not been conducted in the healthy younger subjects. We assumed no abnormalities in blood parameters since our controls had no history of diseases and had not used any medication.

Another limitation is that other studies using skin biopsies in Caucasian subjects included more participants [4, 7, 10]. Moreover, studies which included dark-skinned subjects were larger, but did not conduct skin biopsies [16, 25].

Furthermore, despite the strong correlation of SAF with intrinsic fluorescence and CML with intrinsic fluorescence, correlation of SAF with CML showed a positive trend, but was not significant, probably due to small data numbers, but also possibly due to unsatisfactorily results of SAF for AGE accumulation in very dark skin types. As

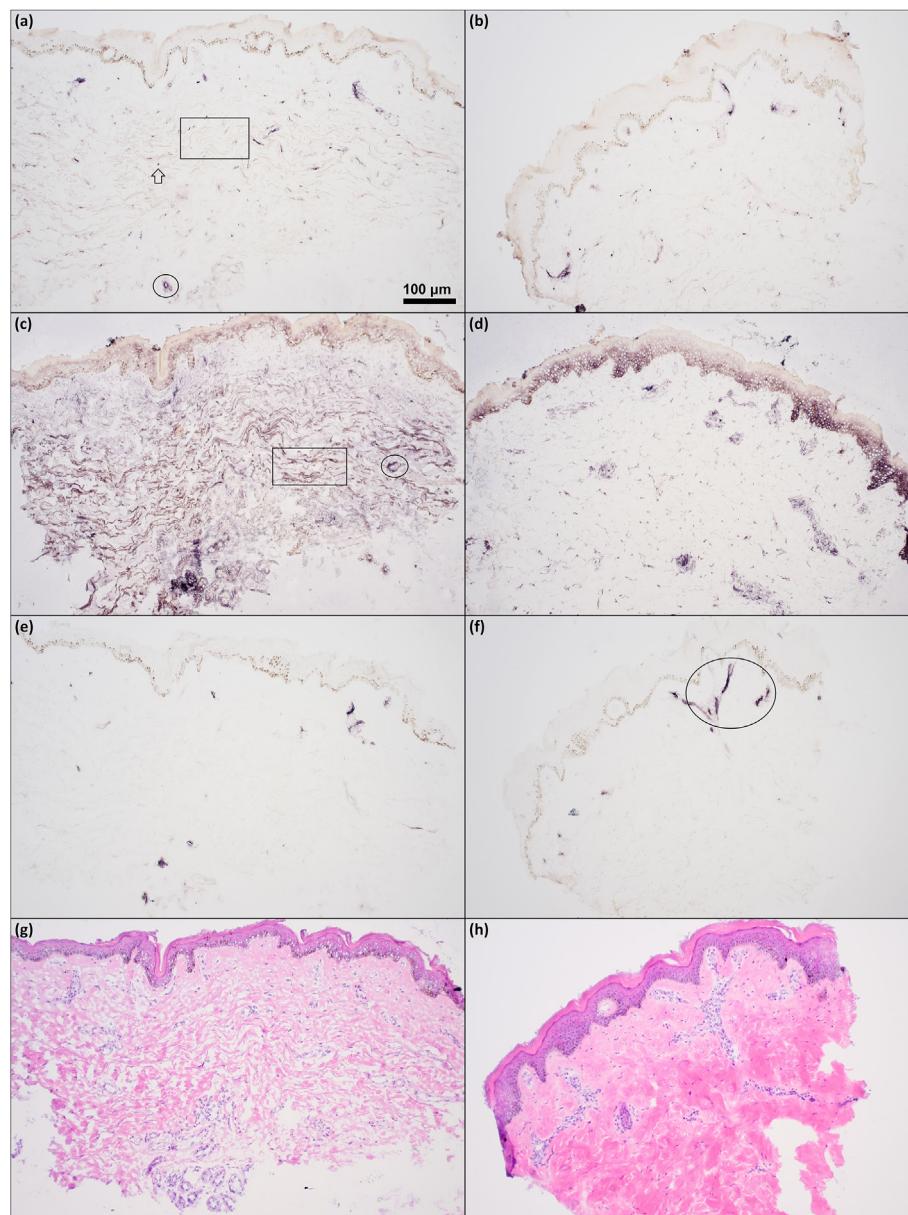


Figure 4. Histology and immunostaining of skin biopsies with anti-AGE antibodies. Anti-CML (a, b), anti-MG-H1 (c, d), negative control (omission of primary antibody) (e, f) and HE stain (g, h) in representative images of older diabetic (a, c, e and g) and healthy young subjects (b, d, f and h). Magnification: $\times 10$. The circle represents a blood vessel, the arrow represents a fibroblast and the square represents collagen. Scale bar = 100 μm .

mentioned before, due to a high intra-individual variance of SAF in dark skin, the interpretation of the SAF levels measured in this study are hampered.

Finally, as mentioned earlier, we compared two small, explicitly contrasting participant groups. Thus, results should be interpreted with caution, but may be useful to calculate sample size in larger studies and encourage further attempts to develop methods for estimating skin AGE levels, using SAF, in dark-skinned subjects.

In conclusion, we are the first to investigate AGEs in skin tissue from dark-skinned subjects, using both invasive and non-invasive techniques. Although non-invasively assessed SAF and invasively measured AGEs are associated with intrinsic fluorescence of the dermis in subjects with dark skin on a group level, the SAF measurement is hampered in individual patients with skin photo type V-VI due to a high intra-individual variation. Further studies to estimate skin AGE levels using SAF in dark-skinned subjects should be encouraged since the exclusion of subjects with skin photo type V-VI is an important limitation.

Declarations

Author contribution statement

I.M. Atzeni: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

P. van der Zee: Performed the experiments; Analyzed and interpreted the data.

A.J. Smit: Conceived and designed the experiments; Analyzed and interpreted the data.

H.H. Pas and G.F.H. Diercks: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, or analysis tools or data.

J.L.J.M. Scheijen and C.G. Schalkwijk: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

J. Boersema, D.J. Mulder: Analyzed and interpreted the data.

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Competing interest statement

The authors declare the following conflict of interests: A.J. Smit is founder and shareholder, and P. van der Zee was involved as a research scientist of DiagnOptics Technologies BV, The Netherlands, manufacturing autofluorescence readers (www.diagnoptics.com).

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2020.e05364>.

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