

Skin Autofluorescence, a Novel Marker for Glycemic and Oxidative Stress-Derived Advanced Glycation Endproducts: An Overview of Current Clinical Studies, Evidence, and Limitations

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ABSTRACT

Background: Advanced glycation endproducts (AGEs) predict long-term complications in age-related diseases. However, there are no clinically applicable markers for measuring AGEs in vivo.

Methods: We have recently introduced the AGE-Reader (DiagnOptics B.V., Groningen, The Netherlands) to noninvasively measure AGE accumulation in the human skin of the forearm, making use of the characteristic autofluorescence (AF) pattern that AGEs encompass. Skin AF is calculated as a ratio of mean intensities detected from the skin between 420–600 nm and 300–420 nm. It correlates with collagen-linked fluorescence and specific skin AGE levels from skin biopsies in diabetes, renal failure, and control subjects. Skin AF levels are increased in patients with diabetes and renal failure and are associated with the presence of vascular complications. Additionally, skin AF is strongly related to the progression of coronary heart disease and mortality, independently of traditional risk factors. Since skin pigmentation might influence skin AF, we have investigated the relation of relative skin reflectance (R%) to skin AF in subjects with varying skin phototypes (SPT).

Results: The data presented in this article suggest that only in subjects with an SPT of V and VI or R% <12%, no reliable measurement can be performed. Therefore, the current prototype of the AGE-Reader is suitable for subjects with SPT I–IV or R% >12%, and more research is needed for a broader application.

Conclusion: The AGE-Reader is useful as a noninvasive clinical tool for assessment of risk for long-term vascular complications in diabetes and in other conditions associated with AGE accumulation.

INTRODUCTION

THE ROLE OF ADVANCED GLYCATION ENDPRODUCTS (AGE) in the pathophysiology of various chronic and age-related diseases has been

scientifically well accepted.¹ Evidence collected in experimental as well as clinical research has provided insight in the several pathways of AGE formation.² Recent developments point out that in addition to the carbohydrate-driven

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Maillard reaction from which AGEs have classically been thought to evolve, other pathways may also be relevant to AGE accumulation. Important intermediates in these other pathways are so-called reactive carbonyl compounds, which may form rapidly under oxidative stress by autooxidation of sugars (and derivatives), but also from peroxidation of fatty acids.³ Because of these two interrelated formation possibilities, glycation and oxidation, the products arising are sometimes referred to as glycoxidation products.⁴ These may therefore include exclusively carbohydrate-derived AGEs, such as pentosidine, both carbohydrate- and lipid-derived structures, such as carboxymethyllysine (CML) and carboxyethyllysine (CEL), or even exclusively lipid-derived compounds, including malondialdehyde-lysine or 4-hydroxynonenal-lysine.² However, apart from glycated hemoglobin (HbA1c), which is an example of an AGE precursor, no biomarker has up until recently been available to easily measure AGEs in clinical studies and routine practice.

In this article, we will describe the importance of measuring AGEs as it has been suggested to be of additional value to current available risk indicators for vascular diseases by representing cumulative glycemic and oxidative stress. Second, we discuss the development of the AGE-Reader (DiagnOptics B.V., Groningen, The Netherlands), which was designed to noninvasively measure AGE accumulation by measuring skin autofluorescence (AF). Finally, we demonstrate the interference of skin color on the measurement with the AGE-Reader, presenting original data.

CLINICAL EVIDENCE FOR THE ROLE OF AGES

Most published clinical studies on AGEs and related compounds have been performed in patients with diabetes and renal failure. In the early 1980s, Monnier and Cerami⁵ postulated that “nonenzymatic browning” (Maillard reaction), which had been known for a long time to occur in foods, could also affect long-lived proteins, such as collagen, *in vivo*. The first method used in humans for detecting AGEs *in vivo* was measuring their characteristic fluorescence in

purified collagen from skin biopsy punches [collagen-linked fluorescence (CLF)]. It was demonstrated that patients with type 1 diabetes appeared to have mean age-adjusted CLF values twice as high as those of control subjects. It also increased with the severity of retinopathy, nephropathy, and arterial and joint stiffness.⁶ In the ensuing years these findings have been confirmed to be also true for other specific AGE compounds measured in skin biopsies, including pentosidine⁷ and CML.^{8,9}

Additionally, in subjects without diabetes with renal failure enhanced deposition of AGEs in skin has also been found.¹⁰ Furthermore, some therapeutic interventions have been shown to decrease levels of skin AGEs. For example, in a Diabetes Control and Complications Trial ancillary study, intensified glycemic control resulted in a decrease in skin glycation products after 6.5 years.¹¹ Also, dialysis in renal failure may result in an attenuation of skin AGE accumulation.¹⁰ In a recent publication, Monnier’s group demonstrated in a 10-year follow-up of former participants from the Diabetes Control and Complications Trial that glycated collagen and CML predict future microvascular events better than HbA1c.¹²

In addition to AGEs linked to long-lived proteins, there is also evidence that circulating AGE-like proteins are involved in diabetes and renal disease. Several serum glycation markers have been associated with diabetic microvascular complications as well as atherosclerotic complications.¹³ In patients with decreased renal function, serum AGEs are even more elevated than in subjects with diabetes having normal renal function. Serum levels of AGEs may rise up to 10-fold in comparison with healthy controls.¹⁴ Although some evidence suggests that serum AGEs correlate with the accumulation of AGEs in tissue,¹⁵ these values may not be a reliable reflection of chronic deposition of AGEs in tissue.^{16,17} This is supported by results from studies demonstrating that serum AGEs are poor prognostic markers for long-term mortality¹⁸ and cardiovascular events.¹⁹

In addition to diabetes and renal disease, AGEs may also play a role in other age-related diseases. For example, AGEs have been histochemically detected in the typical lesions asso-

ciated with age-related macular degeneration,²⁰ Alzheimer's disease and other neurodegenerative diseases,²¹ osteoarthritis,²² and atherosclerosis not associated with diabetes.²³ Plasma AGEs have been shown to be associated with the number of stenotic coronary arteries in subjects without diabetes²⁴ and to positively predict the long-term incidence of cardiovascular mortality in women without diabetes.²⁵ Most convincing evidence for a role of AGEs in cardiovascular disease has been presented by Monnier's group, who demonstrated that CLF measured from skin biopsies predicts atherosclerosis progression measured by coronary calcium scores using computed tomography.²⁶

MEASURING AGES FROM BIOPSIES AND BLOOD SAMPLES

The term AGEs reflects a heterogenic group of compounds, which makes the quantification of AGEs complicated. Although several methods to determine AGE levels have been described, no commercial assay or tool is available yet. Classically, AGEs were determined using their characteristic fluorescence properties (excitation at 370 nm; emission at 440 nm).²⁷ Later on several biochemical detection methods have been developed. For pentosidine, high performance liquid chromatography is considered the most accurate, since enzyme-linked immunosorbent assays suffer from low sensitivity. For CML and CEL, gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry are considered accurate techniques to determine AGE levels.²⁸ High performance liquid chromatography is accurate as well, but is relatively time-consuming.²⁹ Several difficulties with reproducibility and specificity exist when using enzyme-linked immunosorbent assay, especially at low concentrations.³⁰ Currently, a commercial kit from Roche Diagnostics (Basel, Switzerland) is available, presumably circumventing these problems.³¹ Recently, the use of an advanced glycation index, which represents the slope values of AGE fluorescence at several plasma dilutions, was suggested.³² In addition to biochemical assays and fluorescent techniques, several immunohistochemical tech-

niques have been described to determine AGE levels, but are little used in clinical settings.³³ Finally, recent reports have introduced two new concepts of assessing AGEs. First, spectrophotometric and spectrofluorometric techniques—again making use of the fluorescence pattern of AGEs—have been applied for measuring low-molecular-weight AGEs.³⁴ Additionally, a commercial enzyme-linked immunosorbent assay kit (Quantikine, R&D, Minneapolis, MN) has been used in very recent communications to measure the soluble fraction of the receptor for AGEs.^{35,36} Although these studies present interesting data, the association of serum levels of the soluble fraction of the receptor for AGEs with circulating or tissue levels of AGEs has not been assessed yet. Furthermore, there are other ligands associated with this receptor,³⁷ and this method does not take into account the effects of AGEs that are not receptor mediated.

This lack of uniformity in assays should be considered when interpreting data on AGE levels. A second point of concern is that AGEs measured in plasma or serum probably do not reflect the extent of AGE accumulation in tissue, such as the retina, kidney, and vascular wall. Most evidence for the involvement of AGEs in the development of long-term complications has been collected from the measurement of AGEs in skin.^{11,12} An additional limitation of assessing AGEs in plasma or serum is that these are highly subject to kidney function,³⁸ making it difficult to interpret elevated AGE levels in subjects with impaired renal function.³⁹ Therefore, measuring AGEs in tissue (e.g., skin) may better reflect the chronic AGE burden than measuring AGE from serum or plasma.

DEVELOPMENT OF THE AGE-READER

Clinical application of AGE measurements in skin biopsy homogenates has obvious limitations. Additionally, as outlined above, detection of AGEs in serum also has several drawbacks. Therefore, there is a need for a simple and readily available quantification of AGE accumulation in tissue that is suitable for clinical application. The AGE-Reader, which noninva-

sively measures AGE accumulation in skin, meets this need and has proven its value in long-term clinical follow-up studies.⁴⁰

The development of the AGE-Reader was initiated by a serendipitous finding in earlier studies designed to investigate capillary sodium fluorescein leakage in patients with diabetes mellitus as a measure for endothelial dysfunction.⁴¹ Prior to injection of the sodium fluorescein tracer increased levels of fluorescent light emitted from the skin were observed in patients with diabetes compared with controls. This endogenously generated fluorescence is generally referred to as AF. Part of this light can in specific wavelength ranges be attributed to fluorescent AGEs in the different layers of the skin. The principle of skin AF has been used in the field of cancer research, to noninvasively detect local variations in skin AF for tumor growth in tissue.⁴² In ophthalmologic research, AF of lenses of patients with diabetes has been measured. Lens AF is significantly higher in patients with diabetes than in age-matched control subjects and increases significantly with the progression of diabetic retinopathy.^{43–45} However, as a result of the large variation in density and opacity of lens tissue, this method lacks reproducibility. In order to apply the principle of skin AF as a marker for

tissue accumulation of AGE, we designed the AGE-Reader (see Fig. 1a for a photograph). This technique will be outlined in more detail below.

TECHNICAL DETAILS OF THE AGE-READER AND ITS PROTOTYPES

Instrumentation and development of the technique

In contrast to the methods that use AF to detect local variations in tissue, for example, to detect tumors, the purpose of our application is to detect changes that occur in the normal skin with aging and systemic diseases. Therefore, very local measurements are not required. Consequently, the area of the tissue that is detected by the optical fiber should be large enough to prevent gross variations that otherwise might occur on small displacements of the probe, due to follicles, sebaceous glands, and small blood vessels. Therefore, we have chosen to use a fiber that is not in contact with the skin, but that is located at some distance from the skin, leading to an integration area of approximately 0.4 cm². A schematic representation of the instrument is given in Figure 1b. Illuminating light enters the skin almost perpendicularly over an area of ~4 cm² that exceeds the

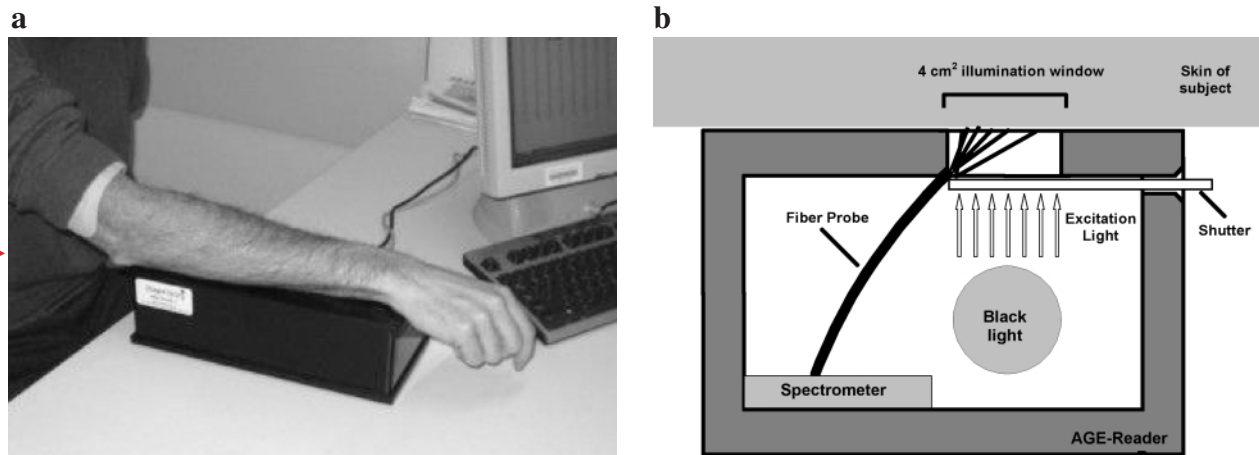


FIG. 1. (a) AGE-Reader in a clinical setting. (b) Schematic view of the AGE-Reader. A black light source is used as excitation light. When the shutter is closed, two calibration measurements are performed: a measurement against a white reflection standard and a dark current measurement. Subsequently, after opening of the shutter, the forearm skin of the subject is illuminated. Emitted light from the skin in the range of 300–600 nm is transmitted by a fiber probe to the integrated spectrometer. Data from the spectrometer are relayed to a computer by USB connection, and the measured spectrum and the skin AF value are displayed on the screen. The total measurement is fully automated and takes approximately 30 s to complete.

detected area in such a way that the results do not depend on the size of the illuminated area. The location of the detection area is thereby chosen in the middle of the illumination area.

For illumination, a black light source is used. In the first prototype, the excitation was in the range 300–420 nm (maximum intensity at 350 nm). In other prototypes the excitation range of 350–420 nm was used (maximum intensity at 370 nm). Figure 2a demonstrates a sample spectrum of the AGE-Reader, and Figure 2b demonstrates fluorescence spectra of controls and patients with diabetes with or without hemodialysis. In most prototype systems spectrometers with an array of charge-coupled device photodetectors were used, which were connected to the AGE-Reader by an optical fiber. In the present AGE-Readers the spectrometers have been integrated in the instrument.

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Measuring procedure for the AGE-Reader

Measurements are performed at the volar site of the forearm, in a semidark environment, preventing surrounding light from interfering with the measurement. Areas with large skin irregularities, such as visible vessels, scars, lichenification, or other skin abnormalities, are not used for measurement.

A measurement with the AGE-Reader consists of two steps. Prior to the subject measurement, two calibration measurements are performed. First, a dark-current measurement is obtained in order to correct for noise from

the charge-coupled device photodetectors. This is subtracted from the spectrum obtained from the subject. Second, reflectance against a white reflection standard, assuming 100% reflectance, is measured. Subsequently, the subject measurement is performed, collecting all light intensity emitted from the skin in the spectrum of 300–600 nm. Light in the range of 420–600 nm is considered to be fluorescence light, whereas light in the 300–420 nm range is caused by skin reflectance. One measurement consists of the average of 50 measurements, to obtain the best reproducible results.

Since skin AF is influenced by variation in skin absorption between subjects, but also by the light intensity of the light source, we present skin AF as relative values, as similarly used by others.⁴⁶ Hence, skin AF is calculated by dividing the average emitted light per nm in the range between 420 and 600 nm by the average excitation light intensity per nm in the range between 300 and 420 nm, expressed as arbitrary units (AU). Finally, relative skin reflectance (R%) is calculated dividing the reflectance in the 300–420 nm range by reflection against the white reflection standard. This gives an indication of the amount of light absorbed by the skin.⁴⁷

Current AGE-Reader

Whereas in many of the prototype instruments the procedures described above had to be carried out manually, the current AGE-Reader allows for a complete automatic mea-

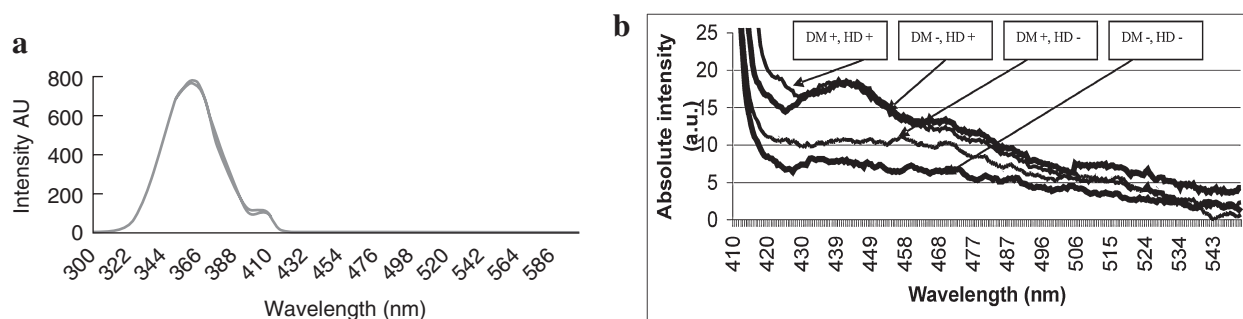


FIG. 2. (a) Light intensities in arbitrary units (AU) from the skin as analyzed by the spectrometer in the 300–600 nm range. The blue line is derived from a patient with diabetes, and the red line is derived from a control subject. AF is calculated by dividing the average light intensity per nm in the range between 420 and 600 nm by the average light intensity per nm in the range between 300 and 420 nm. Reprinted with permission from Meerwaldt et al.⁴⁹ (b) Detail of sample AF spectra in control subjects and patients with diabetes mellitus (DM) and those receiving hemodialysis (HD). a.u. arbitrary units. Reprinted with permission from Meerwaldt et al.⁴⁸

suring procedure within 30 s with immediate presentation of the result. The reflection measurements are performed by using an internal reflection standard, instead of the previously used white Teflon[®] (DuPont, Wilmington, DE) blocks. The operation of the device requires no special training or skills, and needs no special preparation of the subject.

Reproducibility study

Reproducibility was tested by performing repeated AF measurements taken over a single day in 25 healthy subjects and patients with diabetes, which showed a mean relative error in AF of 5%. Reproducibility including seasonal variance among 15 healthy subjects and pa-

tients with diabetes was determined from intra-individual measurements over a longer period of time, also showing a mean relative error of 5%.⁴⁸ The influence of bleaching on skin AF measured with the AGE-Reader seems to be limited, since we did not observe significant decreases in skin AF during continuous measurement over a period of 10 min (authors' unpublished observations).

AGE-READER: CLINICAL STUDIES

Validation

The AGE-Reader was validated in two separate studies, to compare the noninvasively

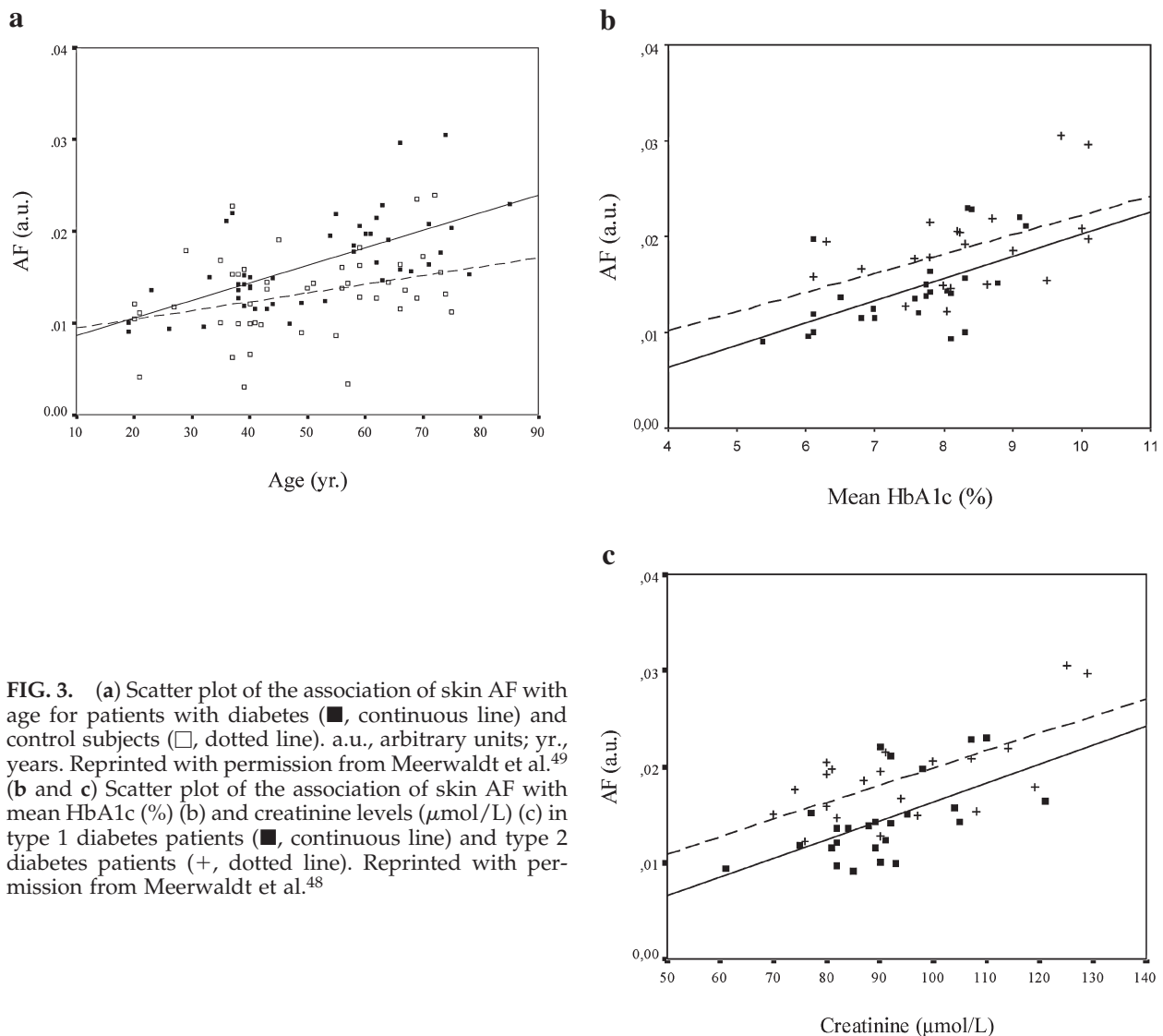


FIG. 3. (a) Scatter plot of the association of skin AF with age for patients with diabetes (■, continuous line) and control subjects (□, dotted line). a.u., arbitrary units; yr., years. Reprinted with permission from Meerwaldt et al.⁴⁹ (b and c) Scatter plot of the association of skin AF with mean HbA1c (%) (b) and creatinine levels (μmol/L) (c) in type 1 diabetes patients (■, continuous line) and type 2 diabetes patients (+, dotted line). Reprinted with permission from Meerwaldt et al.⁴⁸

measured skin AF values with levels of specific AGEs measured from skin biopsy homogenates. The first study, performed with the first prototype of the current AGE-Reader, included patients with diabetes mellitus type 1 and 2 ($n = 46$) and healthy control subjects ($n = 46$).⁴⁹ Skin AF measured at the forearm, lower leg, and abdominal area correlated strongly. Therefore, we decided to perform future measurements only on the forearm, since this is a more practical body site for routine measurement. Mean skin AF was 25% higher in patients with diabetes compared with control subjects. Patients with diabetes did not differ from controls with respect to skin reflectance. The overall variance in skin AF among patients with diabetes could largely ($r = 0.78$, $P < 0.001$) be explained by the independent effects of age ($P < 0.001$; Fig. 3a), mean HbA1c of the previous year ($P < 0.001$; Fig. 3b), and serum creatinine levels ($P = 0.01$; Fig. 3c). In control subjects, the overall variance in skin AF could be explained by the independent effects of age ($P = 0.01$; Fig. 3a) and smoking ($P = 0.04$) ($r = 0.435$, $P = 0.01$). Skin biopsy punches were taken from the same site where the AF measurement was performed. In these skin samples specific AGEs were measured using previously described methods.^{9,50,51} Skin AF correlated strongly with all AGEs measured from the skin samples, including CLF ($r = 0.62$, $P < 0.001$;

Fig. 4a), pentosidine ($r = 0.55$, $P < 0.001$; Fig. 4b), CML ($r = 0.55$, $P < 0.001$), and CEL ($r = 0.47$, $P = 0.002$). The second validation study was performed in patients with end-stage renal disease, treated with hemodialysis. This study confirmed the results from the prior study, showing even stronger correlation with CLF ($r = 0.71$, $P = 0.001$), pentosidine ($r = 0.75$, $P = 0.001$), CML, and CEL (both $r = 0.45$, $P = 0.01$).⁴⁰ Additionally, it was shown that skin AF was 2.4-fold increased in these patients compared with healthy controls, and was independently related to age, dialysis and renal failure duration, presence of diabetes, triglycerides levels, C-reactive protein, and the presence of coronary heart disease. Since skin AF was associated with both carbohydrate-derived AGEs (glycoxidation; CLF and pentosidine) and lipid-derived AGEs (lipoxidation; CML and CEL), we concluded that skin AF can be considered as a noninvasive biomarker for chronic cumulative metabolic stress.

Relation with long-term complications

To study the association of skin AF with peripheral and autonomic nerve abnormalities, skin AF was measured in patients with diabetes with and without neuropathic foot ulcerations.⁵² In neuropathic patients skin AF was 1.5-fold increased compared with patients with

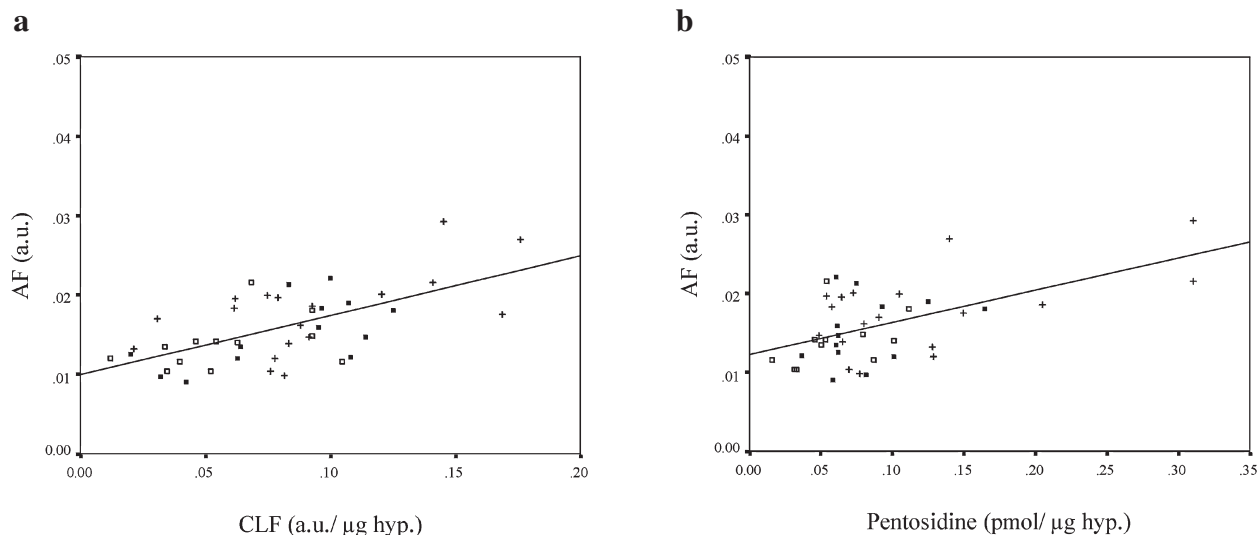


FIG. 4. Scatter plot of the association of skin AF with (a) skin CLF [in arbitrary units (a.u.)/ μg of hydroxyproline (hyp.) content of collagen] and (b) pentosidine (pmol/ μg of hyp.) levels of skin biopsies from Type 1 diabetes patients (■), type 2 diabetes patients (+), and control subjects (□). Reprinted with permission from Meerwaldt et al.⁴⁹

diabetes without neuropathy. Moreover, skin AF was independently associated with severity of ulceration (Wagner score) and several objective markers for nerve damage, including nerve conduction velocity and amplitude, heart rate variability, and baroreflex sensitivity in patients with and without clinically manifested neuropathy. These data suggest that skin AF might detect patients at risk for neuropathy, before its clinical manifestation. In a large population of primary care, well-controlled type 2 diabetes patients, we found that skin AF was substantially higher in patients with micro- as well as macrovascular complications compared with unaffected patients.⁵³

Predictive power of skin AF

Further evidence of the clinical value of skin AF was collected from follow-up studies. In the end-stage renal disease study, skin AF was an independent and strong predictor of 3-year overall [odds ratio (OR) 3.9] and cardiovascular (OR 6.8) mortality.⁴⁰ In a separate study in patients with diabetes having normal renal function, multivariate analysis showed that skin AF replaced HbA1c, triglycerides, and low-density lipoprotein as predictors of 5-year mortality (OR 2.0) (R. Meerwaldt, unpublished data).

Skin AF as a marker of oxidative stress

In several clinical studies, skin AF was positively associated with levels of C-reactive protein, which in atherosclerotic disease may be regarded as an index for oxidative stress-derived inflammation.^{40,54,55} Furthermore, in patients with end-stage renal disease skin AF was inversely related to plasma levels of vitamin C, which is considered to be a potent antioxidant.⁵⁴ In patients with acute ST-elevation myocardial infarction, skin AF is significantly higher compared with patients with stable coronary artery disease, independently of diabetes and smoking. These data suggest that skin AF may be a marker for glycation-derived as well as oxidative stress-derived AGE formation, and may therefore be modulated by acute changes in oxidative stress.⁵⁵

POSSIBLE LIMITATIONS OF THE AGE-READER

As indicated briefly above, skin AF may be influenced by fluorescent compounds (fluorophores) other than AGEs. One potentially confounding fluorophore contained in resident skin cells is the reduced form of NAD(P) [e.g., NAD(P)H; excitation at ~350 nm; emission at 460 nm].⁵⁶ It was reported previously in this journal that in vitro incubation of fibroblasts and adipocytes with a high glucose medium, resulted in increased AF.⁵⁷ However, this was temporary and dropped quickly over time,⁵⁸ and the implications for noninvasive in vivo measurements have not been addressed yet. Furthermore, in a rat model the 360 nm excitation band measured noninvasively at the skin, which is produced by NADH as well as CLF, did not change when hypoxia was induced by pressure occlusion or when the animal was sacrificed. This AF is therefore likely to be mainly from CLF, thus from AGEs, and not from NADH.⁵⁹ Two other fluorophores in the human skin are porphyrins (excitation at ~405 nm; emission at 600 nm) and tryptophan (excitation at ~295 nm; emission at 340–350 nm).⁵⁹ However, their fluorescence spectrum falls outside the spectrum of the AGE-Reader.

In addition to the static fluorescence linked to long-lived proteins, it is likely that skin AF may also be influenced by temporary changes in interstitial and possibly also intracapillary constitution of compounds with fluorescent properties. This phenomenon is supported by the finding that transcapillary and interstitial sodium fluorescein (emission at ~515 nm) diffusion in skin after intravenous injection can be easily detected using videomicroscopy,⁴¹ but also using the AGE-Reader (authors' unpublished data).

In addition to fluorophores in the skin, several chromophores may also induce unwanted variance in skin AF measurement by absorption of excitation but also emission fluorescence light. By far, hemoglobin and melanin are the most important confounders, capable of absorbing light in the 330–400 nm range.⁵⁶ Additionally, epidermal thickness has been proposed as a confounding factor. Sandby-Moller et al.⁶⁰ demonstrated that only skin pigmentation (e.g.,

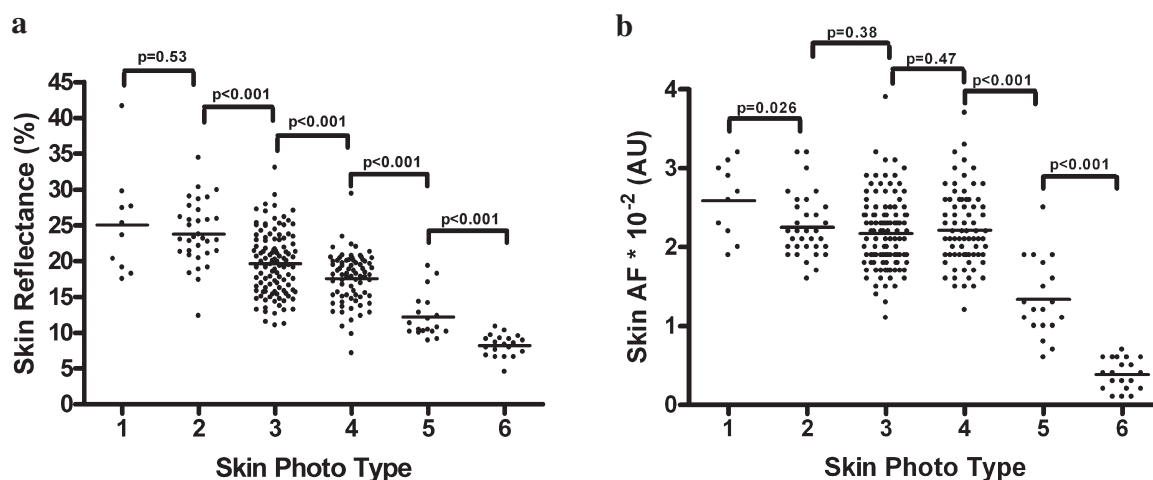


FIG. 5. Scatter plot of the association of skin reflectance (a) and skin AF (b) with SPT. *P* values indicate between-group differences and are tested using Student's *t* test for unpaired variables. Horizontal lines represent mean values. AU, arbitrary units.

melanin) and to a much lesser extent redness (e.g., hemoglobin) significantly influence the amount of emission light from the skin and that the effect of epidermal thickness is negligible. The influence of skin absorption is largely corrected when representing AF as values relative to skin reflectance.⁴⁷ However, there is still some concern that especially skin pigmentation may introduce unwanted intra- and inter-individual variance in measurements.

EFFECTS OF SKIN PIGMENTATION ON SKIN AF AS ASSESSED BY THE AGE-READER

To investigate the behavior of skin AF across different white and dark skin types and to determine the threshold of skin reflectance below which skin AF measurement becomes unreliable, we performed a substudy as part of clinical studies conducted at our hospital. For this study we included 272 subjects, of which 78 had stable coronary artery disease, 158 had subclinical atherosclerosis, and 36 were healthy subjects, in which skin AF and reflectance (R%) were measured using a prototype of the AGE-Reader. All studies were approved by the local ethics committee, and all subjects gave informed consent. No specific inclusion and exclusion criteria were applied for this substudy. To objectively document skin phototype (SPT), ranging from I (pale

skin) to VI (black skin), a questionnaire adapted from Fitzpatrick⁶² was used.

Mean age was 59.3 years (SD 14.1). Subjects with SPT V and VI were significantly older than subjects with SPT I–IV (34.3 ± 15.7 vs. 63.4 ± 8.2 years; $P < 0.001$). SPT was strongly correlated with skin R% ($r = -0.6$; $P < 0.001$; Fig. 5a). From the correlation plots between SPT and skin AF (Fig. 5b), it was evident that SPT V and VI affected skin AF ($r = -0.4$; $P < 0.001$). However, after exclusion of subjects with these skin types, the correlation with skin AF disappeared ($r = -0.07$; $P = 0.30$). The relation between skin AF and skin R% was not strong for $R\% > 13$, but showed a very steep slope at skin $R\% < 12$ –13% (Fig. 6). In the whole group skin

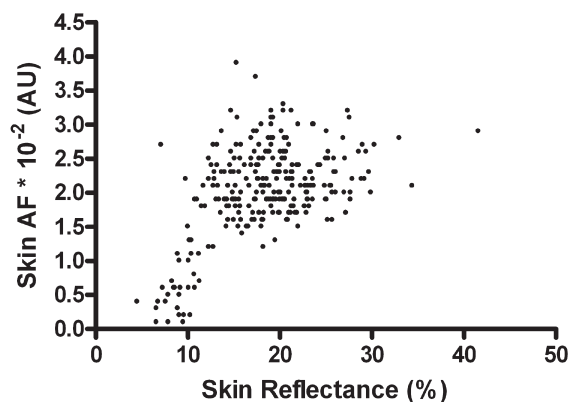


FIG. 6. Scatter plot of the relationship of skin reflectance with skin AF. AU, arbitrary units.

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AF correlated strongly with age ($r = 0.7$; $P < 0.001$). After multivariate regression analysis skin AF was independently associated with skin R% [standardized $\beta = 0.3$; 95% confidence interval (CI), 2.4–4.2; $P < 0.001$] as well as age (standardized $\beta = 0.5$; 95% CI, 0.8–1.1; $P < 0.001$), explaining 55% of its total variance.

From these data, we conclude that skin AF is indeed affected by skin color, as reflected by a significant association with SPT and R%. However, this association was only valid because of inclusion of subjects with a SPT V and VI or R% $< 12\%$. Even in subjects with SPT V, there was a significant correlation with age, giving the option to calculate a correction factor to be able to compare these values with those measured in subjects with a lighter skin color. Unfortunately, the sample size of this study did not allow such an analysis; thus a larger study including more subjects with SPT V and VI is warranted. For the meantime, the AGE-Reader software automatically warns the user not to rely on skin AF values when R% is found to be $< 12\%$ during the measurement. Since some subjects with SPT II–IV may also have R% $< 12\%$, this approach may be more accurate than to rely on SPT. Other approaches to allow for skin AF measurements in subjects with R% $< 12\%$ are under study, including using an actual broadband spectrum of white light as a source for reflectance calculation. This approach may help elicit the transfer function of skin for both the excitation wavelength and the fluorescence, and may provide a better correction for variation based on skin color. Others, using a geometry with multifiber probes, have used an empirical correction using both the reflection coefficients at emission and excitation wavelength.⁶² Additionally, the effect of direct sun exposure on the skin should be addressed, especially in subjects with a lighter skin type. However, this effect may be limited since the seasonal variety of skin AF is $< 6\%$.⁶³

CONCLUSIONS AND FUTURE RESEARCH

With the current published data, we have demonstrated that skin AF is a valuable marker for chronic accumulation of glycation- and ox-

idative stress-derived AGEs. It is associated with chronic complications in diabetes mellitus, including peripheral and autonomic nerve abnormalities, and has independent and incremental power to predict long-term mortality in patients with diabetes and in patients with end-stage renal disease. These studies have been performed in small groups of referral care patients. However, in the near future, follow-up data from a large cohort of approximately 1,000 primary care diabetes patients followed since 2001 will become available. These data will give insight into the capability of skin AF to predict future micro- and macrovascular events and mortality.

Since AGEs may also form from oxidative stress and skin AF is strongly related to skin accumulation of advanced lipoxidation products (e.g., CML and CEL), we are currently investigating the validity of skin AF in diseases that are not classically associated with AGEs, such as stable coronary artery disease and acute coronary syndromes. Preliminary results indeed showed increased skin AF values in these patients.⁵⁵ To enable reliable measurements in subjects with dark skin color, more research is needed.

Based on the cumulative evidence presented in this article, we conclude that measuring skin AF provides an easily applicable and reproducible noninvasive tool to investigate the pathogenetic effects of AGEs in larger populations. Furthermore, skin AF might be a practical tool for estimating risk of complication in diabetes, renal failure, and possibly other diseases.

REFERENCES

1. Smit A, Lutgers H: The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceuticals to reduce AGE accumulation. *Curr Med Chem* 2004;11:2767–2784.
2. Baynes JW, Thorpe SR: Glycoxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* 2000; 28:1708–1716.
3. Miyata T, Ypersele, De Strihou C, Kurokawa K, Baynes JW: Alterations in nonenzymatic biochemistry in uremia: origin and significance of 'carbonyl stress' in long-term uremic complications. *Kidney Int* 1999;55:389–399.
4. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR: The advanced glycation end product,

- Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem* 1996;271:9982–9986.
5. Monnier VM, Cerami A: Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science* 1981;211:491–493.
 6. Monnier VM, Vishwanath V, Frank KE: Relation between complications of Type I diabetes mellitus and collagen-linked fluorescence. *N Engl J Med* 1986;314:403–408.
 7. Sell DR, Lapolla A, Odetti P, Fogarty J, Monnier VM: Pentosidine formation in skin correlates with severity of complications in individuals with long-standing IDDM. *Diabetes* 1992;41:1286–1292.
 8. McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, Lyons TJ: Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 1993;91:2470–2478.
 9. Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 1993;91:2463–2469.
 10. Lee WK, Akyol M, Shaw S, Dominiczak MH, Briggs JD: Kidney transplantation decreases the tissue level of advanced glycosylation end-products. *Nephrol Dial Transplant* 1995;10:103–107.
 11. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glyated collagen products versus HbA_{1c} as markers of diabetic complications. DCCT Skin Collagen Ancillary Study Group. *Diabetes Control and Complications Trial*. *Diabetes* 1999;48:870–880.
 12. Genuth S, Sun W, Cleary P, Sell DR, Dahms W, Malone J, Sivitz W, Monnier VM; DCCT Skin Collagen Ancillary Study Group: Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the Diabetes Control and Complications Trial and Epidemiology of Diabetes Interventions and Complications participants with Type 1 diabetes. *Diabetes* 2005;54:3103–3111.
 13. Vlassara H, Uribarri J: Glycoxidation and diabetic complications: modern lessons and a warning? *Rev Endocr Metab Disord* 2004;5:181–188.
 14. Kalousova M, Zima T, Tesar V, Stipek S, Sulkova S: Advanced glycation end products in clinical nephrology. *Kidney Blood Press Res* 2004;27:18–28.
 15. Kanauchi M, Nishioka H, Dohi K: Serum levels of advanced glycosylation end products in diabetic nephropathy. *Nephron* 2001;89:228–230.
 16. Dorrian CA, Cathcart S, Clausen J, Shapiro D, Dominiczak MH: Factors in human serum interfere with the measurement of advanced glycation endproducts. *Cell Mol Biol* 1998;44:1069–1079.
 17. Hricik DE, Wu YC, Friedlander MA, Schulak JA: Disparate changes in plasma and tissue pentosidine levels after kidney and kidney-pancreas transplantation. *Clin Transplant* 1996;10:568–573.
 18. Schwedler SB, Metzger T, Schinzel R, Wanner C: Advanced glycation end products and mortality in hemodialysis patients. *Kidney Int* 2002;62:301–310.
 19. Stein G, Busch M, Miller A, Muller A, Wendt T, Franke C, Niwa T, Franke S: Are advanced glycation end products cardiovascular risk factors in patients with CRF? *Am J Kidney Dis* 2003;41(3 Suppl 1):S52–S56.
 20. Stitt AW: Advanced glycation: an important pathological event in diabetic and age related ocular disease. *Br J Ophthalmol* 2001;85:746–753.
 21. Sasaki N, Fukatsu R, Tsuzuki K, Hayashi Y, Yoshida T, Fujii N, Koike T, Wakayama I, Yanagihara R, Garruto R, Amano N, Makita Z: Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. *Am J Pathol* 1998;153:1149–1155.
 22. Verzijl N, Bank RA, TeKoppele JM, DeGroot J: AGE-ing and osteoarthritis: a different perspective. *Curr Opin Rheumatol* 2003;15:616–622.
 23. Stitt AW, He C, Friedman S, Scher L, Rossi P, Ong L, Founds H, Li YM, Bucala R, Vlassara H: Elevated AGE-modified ApoB in sera of euglycemic, normolipidemic patients with atherosclerosis: relationship to tissue AGEs. *Mol Med* 1997;3:617–627.
 24. Kanauchi M, Tsujimoto N, Hashimoto T: Advanced glycation end products in nondiabetic patients with coronary. *Diabetes Care* 2001;24:1620–1623.
 25. Kilhovd BK, Juutilainen A, Lehto S, Ronnema T, Torjesen PA, Birkeland KI, Berg TJ, Hanssen KF, Laakso M: High serum levels of advanced glycation end products predict increased coronary heart disease mortality in nondiabetic women but not in nondiabetic men: a population-based 18-year follow-up study. *Arterioscler Thromb Vasc Biol* 2005;25:815–820.
 26. Monnier VM: Skin collagen fluorescence predicts coronary artery calcium deposition in the Epidemiology of Diabetes Intervention and Complications (EDIC) study [abstract]. *Diabetes* 2004;53:A77.
 27. Monnier VM, Vishwanath V, Frank KE, Elmets CA, Dauchot P, Kohn RR: Relation between complications of type I diabetes mellitus and collagen-linked fluorescence. *N Engl J Med* 1986;314:403–408.
 28. Niwa T: Mass spectrometry in the search for uremic toxins. *Mass Spectrom Rev* 1997;16:307–332.
 29. Miyata T, Ueda Y, Shinzato T, Iida Y, Tanaka S, Kurokawa K, van Ypersele de Strihou C, Maeda K: Accumulation of albumin-linked and free-form pentosidine in the circulation of uremic patients with end-stage renal failure: renal implications in the pathophysiology of pentosidine. *J Am Soc Nephrol* 1996;7:1198–1206.
 30. Dorrian CA, Cathcart S, Clausen J, Shapiro D, Dominiczak MH: Factors in human serum interfere with the measurement of advanced glycation endproducts. *Cell Mol Biol (Noisy-le-grand)* 1998;44:1069–1079.
 31. Schiel R, Franke S, Appel T: Improvement of the quality of diabetes control and decrease in the concentra-

- tions of AGE-products in patients with type 1 and insulin-treated type 2 diabetes mellitus-results from a 10 year-prospective, population-based survey on the quality of diabetes care in Germany (JEVIN). *Eur J Med Res* 2004;9:391-399.
32. Sampathkumar R, Balasubramanyam M, Rema M, Premanand C, Mohan V: A novel advanced glycation index and its association with diabetes and microangiopathy. *Metabolism* 2005;54:1002-1007.
 33. Rocken C, Kientsch-Engel R, Mansfeld S, Stix B, Stubenrauch K, Weigle B, Buhling F, Schwan M, Saeger W: Advanced glycation end products and receptor for advanced glycation end products in AA amyloidosis. *Am J Pathol* 2003;162:1213-1220.
 34. Wrobel K, Wrobel K, Garay-Sevilla ME, Nava LE, Malacara JM: Novel analytical approach to monitoring advanced glycosylation end products in human serum with on-line spectrophotometric and spectrofluorometric detection in a flow system. *Clin Chem* 1997;43:1563-1569.
 35. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D: Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol* 2005;25:1032-1037.
 36. Geroldi D, Falcone C, Emanuele E, D'Angelo A, Calcagnino M, Buzzi MP, Scioli GA, Fogari R: Decreased plasma levels of soluble receptor for advanced glycation end-products in patients with essential hypertension. *J Hypertens* 2005;23:1725-1729.
 37. Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP: AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. *Cardiovasc Res* 1998;37:586-600.
 38. Stam F, Schalkwijk CG, van Guldener C, ter Wee PM, Stehouwer CDA: Advanced glycation end-product peptides are associated with impaired renal function, but not with biochemical markers of endothelial dysfunction and inflammation, in non-diabetic individuals. *Nephrol Dial Transplant* 2006;21:677-682. Epub 2005 Dec 5.
 39. Deppisch R, Beck W, Ritz E, Henle T, Hergesell O, Hensch GM: Advanced glycated end-products (AGE) during haemodialysis treatment: discrepant results with different methodologies reflecting the heterogeneity of AGE compounds. *Nephrol Dial Transplant* 1999;14:1968-1975.
 40. Meerwaldt R, Hartog JW, Graaff R, Huisman RJ, Links TP, den Hollander NC, Thorpe SR, Baynes JW, Navis G, Gans RO, Smit AJ: Skin autofluorescence, a measure of cumulative metabolic stress and advanced glycation end products, predicts mortality in hemodialysis patients. *J Am Soc Nephrol* 2005;16:3687-3693.
 41. Jager J, Oomen PH, Sluiter WJ, Reitsma WD, Smit AJ: Improved reproducibility of the 'large-window' method of assessing transcapillary and interstitial fluorescein diffusion in the skin in healthy subjects and in subjects with insulin-dependent diabetes mellitus. *Int J Microcirc Clin Exp* 1997;17:150-158.
 42. Utzinger U, Richards-Kortum RR: Fiber optic probes for biomedical optical spectroscopy. *J Biomed Opt* 2003;8:121-147.
 43. Koefoed Theil P, Larsen M, Lund-Andersen H, Hansen T, Pedersen O: Lens autofluorescence is increased in newly diagnosed patients with NIDDM. *Diabetologia* 1996;39:1524-1527.
 44. Mota MC, Pereira P, Burrows H, Morgado AM, Matos A: Evaluation of a non-invasive fluorescence technique as a marker for diabetic lenses in vivo. *Graefes Arch Clin Exp Ophthalmol* 1999;237:187-192.
 45. Sato E, Mori F, Igarashi S, Abiko T, Takeda M, Ishiko S, Yoshida A: Corneal advanced glycation end products increase in patients with proliferative diabetic retinopathy. *Diabetes Care* 2001;24:479-482.
 46. Coremans JM, Ince C, Bruining HA, Puppels GJ: (Semi-)quantitative analysis of reduced nicotinamide adenine dinucleotide fluorescence images of blood-perfused rat heart. *Biophys J* 1997;72:1849-1860.
 47. Na R, Stender IM, Henriksen M, Wulf HC: Autofluorescence of human skin is age-related after correction for skin pigmentation and redness. *J Invest Dermatol* 2001;116:536-540.
 48. Meerwaldt R, Links T, Graaff R, Thorpe SR, Baynes JW, Hartog J, Gans R, Smit A: Simple noninvasive measurement of skin autofluorescence. *Ann NY Acad Sci* 2005;1043:290-298.
 49. Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, Thorpe SR, Baynes JW, Gans RO, Smit AJ: Simple non-invasive assessment of advanced glycation endproduct accumulation. *Diabetologia* 2004;47:1324-1330.
 50. Ahmed MU, Brinkmann FE, Degenhardt TP, Thorpe SR, Baynes JW: N-epsilon-(Carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 1997;324:565-570.
 51. Maekawa T, Rathinasamy TK, Altman KI, Forbes WF: Changes in collagen with age. I. The extraction of acid soluble collagens from the skin of mice. *Exp Gerontol* 1970;5:177-186.
 52. Meerwaldt R, Links TP, Graaff R, Hoogenberg K, Lefrandt JD, Baynes JW, Gans RO, Smit AJ: Increased accumulation of skin advanced glycation end-products precedes and correlates with clinical manifestation of diabetic neuropathy. *Diabetologia* 2005;48:1637-1644.
 53. Lutgers HL, Links TP, Graaff R, Bilo HJG, Gans RO, Smit AJ: Skin AGE-levels are increased in type 2 diabetes mellitus with complications [abstract]. *Diabetologia* 2005;48:A419.
 54. Hartog J, de Vries A, Lutgers H, Meerwaldt R, Huisman RM, van Son WJ, de Jong PE, Smit AJ: Accumulation of advanced glycation end products, measured as skin autofluorescence, in renal disease. *Ann NY Acad Sci* 2005;1043:299-307.
 55. Mulder DJ, van Haelst PL, Graaff R, Smit AJ, Gans RO, Zijlstra F: Skin autofluorescence is an independent marker for acute myocardial infarction. *Circulation* 2005;112:II-371.

56. Kollias N, Zonios G, Stamatatos GN: Fluorescence spectroscopy of skin. *Vibrational Spectrosc* 2002;28:17–23.
57. Evans ND, Gnudi L, Rolinski OJ, Birch DJ, Pickup JC: Non-invasive glucose monitoring by NAD(P)H autofluorescence spectroscopy in fibroblasts and adipocytes: a model for skin glucose sensing. *Diabetes Technol Ther* 2003;5:807–816.
58. Evans ND, Gnudi L, Rolinski OJ, Birch DJ, Pickup JC: Glucose-dependent changes in NAD(P)H-related fluorescence lifetime of adipocytes and fibroblasts in vitro: potential for non-invasive glucose sensing in diabetes mellitus. *J Photochem Photobiol B* 2005;80: 122–129.
59. Kollias N, Gillies R, Moran M, Kochevar IE, Anderson RR: Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging. *J Invest Dermatol* 1998;111:776–780.
60. Sandby-Moller J, Poulsen T, Wulf HC: Influence of epidermal thickness, pigmentation and redness on skin autofluorescence. *Photochem Photobiol* 2003;77: 616–620.
61. Fitzpatrick TB: The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol* 1988;124:869–871.
62. Hull E, Ediger M, Unione A, Deemer E, Stroman M, Baynes J: Noninvasive, optical detection of diabetes: model studies with porcine skin. *Optics Express* 2004;12:4496–4510.
63. Graaf R, Meerwaldt R, Lutgers HL, Baptist R, de Jong ED, Zijp JR, Links TP, Smit AJ, Rakhorst G: Instrumentation for the measurement of autofluorescence on the human skin. *Proc SPIE* 2005;5692:111–118.

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