**Skin Autofluorescence, A Non-Invasive Marker of Advanced Glycation End Products: Clinical Relevance and Limitations**

**Running Title:** Skin autofluorescence, Advanced Glycation End Products, clinical relevance and challenges

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**ABSTRACT**

Advanced glycation end products (AGEs) are protein bound compounds derived from glycaemic and oxidative stress that contain fluorescent properties, which can be non-invasively measured as skin autofluorescence (SAF) by the AGE-Reader. SAF has been demonstrated to be a biomarker of cumulative skin AGEs (SAGEs) and potentially may be a better predictor for the development of chronic complications and mortality in diabetes than glycatedhaemoglobinA1c (HbA1c).  However, there are several confounding factors that should be assessed prior to its broader application: these include presence of other fluorescent compounds in the skin that might be measured (e.g. fluorophores), skin pigmentation and use of skin creams. The aim of this article is to provide a theoretical background of this newly developed method, evaluate its clinical relevance, and discuss the potential confounding factors that need further analysis.

**Keywords:** Advanced glycation end products, skin autofluorescence, AGE-Reader, diabetes, cardiovascular risk, confounding factors

**Abbreviations:** AGEs, advanced glycation end products; AU, arbitrary units; CAD, carotid artery disease; CEL, N-carboxyethyl-lysine; CML, N-carboxymethyl-lysine; CV, cardiovascular; DHA, dihydroxyacetone; DM, diabetes mellitus; HbA1c glycatedhaemoglobin; HR, hazard ratio; MACE, major adverse cardiac event; OR, odds ratio; PAD, peripheral artery disease; SAF, skin autofluorescence; SAGES, skin advanced glycation end products; sHRsubproportional hazard ratio; SR%, skin reflectance percentage; STEMI, ST-elevation myocardial; T1DM, Type 1 Diabetes Mellitus; T2DM, Type 2 Diabetes Mellitus; UKPDS, United Kingdom Prospective Diabetes Study; yr, year;

**INTRODUCTION**

The significant prevalence of diabetes mellitus (DM) and associated long-term complications have emerged as a global public health crisis in the recent years. It is estimated that approximately 552 million people worldwide will be affected with diabetes by 2030, predominantly in developing countries.1 Chronic hyperglycaemia is the main cause of diabetic complications and has adverse effects in different tissue and organ systems, which leads to a complex cascade of events that are not fully understood.2 One of these events is the increased production of AGEs in tissues. These are a group of heterogeneous compounds produced by non-enzymatic attachment of glucose to proteins, lipids or nucleic acids through the Maillard reaction, which significantly alters their function by affecting molecular conformation, enhancing cross-linking, changing enzyme activity and reducing receptor recognition.2The accumulation of AGEs result in adverse structural and functional modifications of tissues as such AGEs are thought to play a central role in the development of cardiovascular and renal complications associated with diabetes 3-5

Since specific SAGEs exhibit intrinsic fluorescence properties, they can be measured by SAF using an AGE Reader (AGE Reader, DiagnOptics, Groningen, the Netherlands), which is a simple and non-invasive technique.6 Validation studies have provided consistent evidence about the significant association of SAF with AGEs content in skin biopsies. In particular, SAGEs content accounted for up to 76% of the variance in the SAF levels in a meta-analysis of three validation studies,6-8 indicating that SAF can act as a measurable variable of SAGEs accumulation.  Furthermore, SAGEs have been demonstrated to be a more significant predictor of diabetic complications and mortality over time (5 - 10 years) than the short-term glycaemic memory reflected by HbA1c (3-6 months).9-11

Although, SAGEs assessment has been revealed to be a valuable non-invasive tool to estimate future disease risk, it is important to identify SAF confounding factors prior to application in the clinical setting. The effects of other skin fluorophores, skin pigmentation and the use of emollients have been reported on the validity of measurements.12-14 This article provides a background on SAGEs formation, discuss the clinical relevance of this tool and critically review factors influencing the measurements and thus require further assessment.

**FORMATION OF ADVANCED GLYCATION END PRODUCTS**

Chronic hyperglycaemia enhances the production and accumulation of AGEs endogenously and exogenously as a consequence of oxidative and non-oxidative reactions of amino acids and reducing sugars in the Maillard reaction (Figure 1).15 Protein glycation is primarily initiated when the carbonyl groups of reducing sugars, such as glucose interact non-enzymatically with the reactive amino (α-NH2) groups of proteins ( lysine or arginine residues). Consequently, this interaction forms an unstable aldimine compound, a Schiff base. This reaction is relatively fast and is highly reversible, depending on substrate concentrations and incubation time. The Schiff base can be rearranged producing a stable Amadori product (e.g HbA1c), which accumulates on both short-lived and long-lived proteins over a period of several weeks.16 The Amadori product undergoes oxidative degradation to generate highly reactive intermediate dicarbonyl compounds, known as  α-oxoaldehydes ( glyoxal, methyglyoxal, deoxyglucosones) (Figure 1) that interact again with free amino groups of proteins. Then, complex rearrangements of condensation, dehydration, hydrolysis or cross-linking reactions occur so highly heterogeneous, often fluorescent, insoluble and irreversible group of AGEs are formed, which accumulate and damage long-lived proteins such as extracellular matrix collagens.15 16

The accumulation of most AGEs occurs by intracellular glycation, cross-link formation in the extracellular matrix and interaction with specific cellular receptors.16 So far, a number of AGEs have been identified in the skin and these can be distinguished according to their fluorescence properties and cross-linking structures. These are fluorescent cross-link AGEs (pentosidine), non-fluorescent protein adducts (N-carboxymethyl-lysine (CML), N-carboxyethyl-lysine (CEL), glucosepane and fructose-lysine), and non-fluorescent protein cross-links ( glyoxal-lysine dimer and methylglyoxal-lysine dimer).18

**THE PRINCIPLES BEHIND THE MEASUREMENT OF SKIN ADVANCED GLYCATION END PRODUCTS**

Most AGEs that are evaluated *in vivo* or *in situ* can be detected in different specimen types and work as biomarkers of chronic and age-related diseases.18 Several biochemical and immunochemical assays have been developed to determine levels of circulating AGEs.  However, due to the heterogeneity of AGEs, cost, lack of reproducibility and high physiological fluctuations**,**12 an alternative assessment of AGEs accumulation in the tissue is beneficial.

Since 2004, a simple, accurate and non-invasive screening technique SAF has been widely used in clinical trials to determine (in real time) tissue AGE accumulation in patients with diabetes, cardiovascular and renal complications: The AGE Reader (Figure 2).6 SAF is directly correlated with both fluorescent (pentosidine) and non-fluorescent AGEs (CML and CEL) in the skin biopsies and so can be applied as an estimation method of SAGEs accumulation.6

This device illuminates a subject’s dominant lower forearm avoiding skin abnormalities.  When the device is turned on, an UV tube emits ultraviolet light with peak intensity at 370 nm, which excites the AGEs in the skin that have characteristic autofluorescence properties in a frequency range of 300-420 nm. The autofluorescent light is measured with a spectrophotometer in the range of 300-600 nm. Normally, for each SAF value, three consecutive records are performed at three different sites of the same forearm, within a period of approximately 2 minutes. SAF is expressed in arbitrary units (AU) as the ratio between the emission light intensity in the 420-600 nm range by the excitation light intensity between 300-420 nm multiplied by 100 to compensate for the effect of skin pigmentation on autofluorescence by light absorption. Data from the spectrometer provides the SAF value which is displayed on the screen immediately, together with an age-corrected reference value. 19 The intra-individual and inter-day precision of this instrument has been tested previously  in diabetic participants, giving  a mean relative error of 4.2 - 5%.20

The skin reflectance percentage (SR%), is  automatically expressed by the average intensities of the UV light reflected from the skin from a white reference standard in the 300-420 nm range. This calculation allows the reliable measurement of SAF independent of skin colour if the SR% is between 6% and 10%, which covers Fitzpatrick skin colourclasses I-IV. However, when SR< 6% (Fitzpatrick V-VI) no measurements are given by the AGE-Reader.13 The Fitzpatrick scale is a semi-quantitative classification for assessing patient’s skin colour by cutaneous response to UV 22 (Table 1).

**CLINICAL RELEVANCE**

**Diabetes and Chronic Complications**

There is consistent evidence that SAF is directly correlated with DM and development of diabetic complications (Table 2).In a cross-sectional study, SAF values were considerably higher in type 2 diabetes (T2DM) patients (n=973) than healthy subjects (n=231) across age groups. 23 The SAF values were increased in the presence of diabetes microvascular and macrovascular complications compared with those without complications ( 3.12 AU and 2.57 AU, respectively; P <0.001), independently of recent HbA1c levels.This agreed with similar SAF values reported from a prospective study in a multi-centre secondary care setting.3

The predictive value of SAF for the development of microvascular complications was assessed by T2DM patients (n=881) with good metabolic control of HbA1c 6.6%. After a mean follow-up period of 3 years, SAF at baseline revealed to be a significant predictive value for the progress of microvascular complications, predominantly for neuropathy and nephropathy but not for retinopathy.11 These associations were also observed in a study performed in type 1 DM (T1DM) patients, where SAF levels were correlated with nephropathy and neuropathy but again not with retinopathy.24 Nevertheless, SAF has demonstrated to be significantly correlated with diabetic neuropathy and retinopathy and with past long-term glycemic control in a study of T1DM Japanese subjects over 15 years.25 Therefore, suggesting that SAF might be an important early surrogate marker for the harmful consequences of hyperglycemia and oxidative stress over time, where it may be superior to other risk predictors of diabetes (or chronic complications) such as HbA1c and diabetes duration.

**Cardiovascular Risk and Mortality**

SAF is an important marker for the induction and development of vascular complications that can predict cardiovascular risk and death (Table 2), when SAF values > 2.0 AU over 5 years.10 Cardiovascular (CV) risk is conventionally assessed according to age, blood pressure, lipid profile, smoking and DM 26, however SAF measurements have shown additional value to the United Kingdom Prospective Diabetes Study (UKPDS) risk engine for cardiovascular risk assessment. 27 In this study, 25% of patients with a low UKPDS risk score (<10%) were reclassified at high risk to develop fatal and non-fatal cardiovascular events after the addition of SAF and vice-versa. Moreover, SAF was able to predict those at high risk for fatal cardiovascular event in the next 10 years within the group with a high risk score (>10%).27

Recently, SAF values revealed to be elevated in patients with coexistent carotid artery disease (CAD) and peripheral artery disease (PAD) (3.28 AU) compared to subjects with only CAD (2.66 AU).28 Therefore, indicating that SAF can potentially be a specific marker of atherosclerotic disease. Subjects with PAD showed higher SAF values than their control counterparts ( 2.77 AU versus 2.44 AU; p<0.001) independently of cardiovascular risk factors and comorbidity.29 Moreover, patients with PAD (n=252), revealed that SAF was independently related with all-cause mortality and fatal or non-fatal major adverse cardiovascular events 4 and predicted lower limb amputation 30 during a follow-up period of 5 years irrespective of DM and  other CV risk factors.

**CHALLENGES AND FUTURE PERSPECTIVES**

As understanding of the prominent role of SAGEs accumulation and clinical consequences, the AGE- Reader contains several limitations that must be addressed to improve its relevance in the clinical setting.

**Skin fluorophores**

SAF assessment may not represent only the SAGEs content. The presence of endogenous fluorescent signals from skin fluorophores (e.g. Nicotinamide adenine dinucleotide) that are within the same excitation and emission ranges of the AGE-Reader ( 350-410 nm and 420-600 nm respectively) restrict the recognition of specific fluorophores on the total fluorescence signal.13However, 76% of the variance in the SAF signal correlates with specific SAGEs content 6-8 and the broad excitation peak of this device is ideal to assess the development of chronic complications in diabetes.31 Therefore, indicating  that less costly spectrometers or simple light sources can be implemented in this tool and so its application in the primary care can be extended.

**Skin pigmentation**

One important improvement for this instrument is to assess measurements in participants with darker skin pigmentation. The reliable analysis of SAGEs in darker skin subjects       (SR< 10%) has been particularly hindered, because strongly pigmented skin tends to absorb excitation light and so the identification of increased SAF values can’t be predicted.12 32

Studies reported that this may be due to distinct absorption of light by skin compounds or scattering effects.12 33 The epidermis and dermis have ultraviolet radiation and visible region absorbing endogenous chromophores, melanin and hemoglobin, that absorb UV-A light within the total reflected wavelength range between 320-420 nm.13 In particular, melanin has been shown to have a photoprotective role by acting as a physical barrier against UV radiation and as an absorbent chromophore that decreases the penetration of UV through the epidermis.34 The total melanin content in the epidermis was demonstrated to be higher in darker skin subjects, while those with Asian and fairer skin differed by only 2-fold.34 Indeed, SAF values between age-matched healthy Dutch and Chinese subjects revealed to be similar with SR> 10% (P=0.253)**.**32 35

Currently, there is limited data about the SAF values in non-Caucasian patients with diabetes or diabetic complications.  Most publications reporting other ethnic groups included East Asian subpopulations, predominantly from Japan or China.25,36-37 Therefore, it is essential to assess SAF levels in dark skinned subjects since they are the most prevalent skin type of the world and represent the group with highest incidence of diabetes.38Koetsier*et al*. validated an adjusted algorithm to determine the UV light absorption in the spectral regions of melanin.13 Although, these corrections have so far extended the range of SAF detection in darker skin subjects ( up to Fitzpatrick class IV ) with SR ≥ 6 %, they were only developed in healthy subjects and have not been proved reliable in diabetes or diabetes complications from other ethnicities. Accordingly, a study involving a Saudi population (n=1999)revealed that the SAF risk score model may be not suitable for subjects with higher SAF values than those previously reported in Caucasian subjects, 39 because it underestimated subjects with low and high SR at high risk of developing cardiovascular disease.39 This is therefore an urgent need for AGE-Reader risk estimations to be tested in different ethnic populations.

**Use of skin creams**

SAF is strongly influenced by the application of different skin creams, leading to imprecise elevated SAF values and decreased SR%. This effect may be due to the creams’ fluorescent characteristics from day cream or sunscreens that absorb ultraviolet radiation in order to protect the skin from adverse health effects. Day cream and sunscreen applied in the forearm provided 139% and 111% rise in the SAF measurements compared to 18% obtained by body lotion.14 Moreover, use of self-browning dihydroxyacetone (DHA) cream results in a chemical reaction with the stratum corneum to cause brown discoloration when applied to the skin and elevated absorption of UVA light, with a significant SAF increase of 298%.14

Washing the forearm with soap or using alcohol swabs does not reverse the effect of particular type of creams and can persist for many days. For example, sunscreen persisted for 4 days while self-browning cream 2 weeks,14 showing it is important to take these potential error sources into consideration in order to prevent discrepancies in the SAF measurements. The different prevalence of skin creams used between men and women may account for gender differences in SAGEs as previously reported.40This gender bias raises questions about the values obtained for cardiovascular and diabetes mortality between genders as estimated by the World Health Organization country profile.38

Thus far, the AGE-Reader user manual suggests not conducting SAF measurements for at least 2 days after use of self-tanning agents on the forearm. These control measures have the potential to guarantee accuracy in the predictive values of SAF, which may be greater than those previously reported and where these precautions may not have been followed. Nevertheless, these measures do not necessarily detract from its application in the future. Currently, additional improvements in the AGE-Reader software to limit significant fluctuations of SAF values are in development. 14

**CONCLUSION**

In conclusion, AGE accumulation is demonstrated to play a pathophysiological role in the development of chronic complications in diabetes, including microvascular, macrovascular, cardiovascular complications and overall mortality. Most importantly, SAF assessment revealed to be an important biomarker of AGEs burden and a better predictor of diabetes complications because it represents a more “long-term memory” of cumulative metabolic stress than HbA1c or other conventional risk factors (i.e. smoking and blood pressure).     This non-invasive tool is currently used for research purposes and whether it would be beneficial in daily clinical practice requires further studies, but its advantages constitutes a consistent perspective of a variable suitable for monitoring interventions and limit the incidence of diabetes complications in the future. Thus far, SAF measurements contain several technical issues that are still being improved. Therefore, further research to expand the application of this technique is crucial as this is a practical, relatively inexpensive, and non-invasive procedure available to be used in outpatient clinics.

**Main messages**

* The AGE-Reader is a non-invasive, practical and mobile device with low educational or training input.
* SAF assessment extends history of hyperglycaemia compared to conventional HbA1c
* SAF is a measure of SAGEs, which may be useful in large scale studies and clinical setting, mostly when follow-up measurements are required to predict progression of DM related complications and  allow targeting of therapy
* SAF measurements are strongly influenced by the optical properties of the skin and so it is crucial to adjust measurements for these confounding factors to be able to compare results between subjects and different ethnicities.

**Current research questions**

* Does the accumulation of AGEs represent the pathology of diabetes or other pathological mechanisms?
* Does SAF also detects non-fluorescent AGEs as depicted by skin biopsies?
* What will be the reasonable time frame to assess improvements of the AGEs levels after treatment?
* Is SAF a suitable method to predict diabetic complications in non-caucasian ethnicities?

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**Self-assessment Questions**

1. The AGE Reader provides a rapid assessment of diabetes complications and cardiovascular risk prediction or mortality.
2. The reported SAF reference values in Chinese subjects is significantly higher than Caucasian subjects
3. Darker skin pigmentation can potentially affect SAF measurements
4. The effect of skin creams on SAF measurements may not fully be reversed by cleaning the forearm with water or alcohol swab.
5. The fluorescent characteristics detected by the AGE Reader represent only SAGEs content.

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**Answers**

1. True

2. False

3. True

4. True

5. False

**Figure 1** The Maillard reaction. Adapted from Nagai *et al*.17 This process involves three stages. In the early stage, glucose reacts with a free amino group of a protein to generate Schiff base that is subjected to further rearrangements to form Amadori product. These both processes are reversible (red arrows).  Then, reactive dicarbonyl compounds are formed at the advanced stage by either from the degradation of Amadori product or metal-catalyzed autoxidation of glucose (blue arrows). Subsequently, irreversible AGEs are formed.

**Figure 2** Schematic view of the AGE Reader. Adapted from Gerrits*et al*.21 Firstly, two calibration records are performed when the shutter is closed. One from white reflection standard and another from a dark current measurement. The light source will excite light (purple arrows) when the shutter is opened to illuminate the patient’s forearm skin through the illumination window. Subsequently, the emitted light from the skin is transferred through the fiber probe to the integrated spectrometer in order to transmit the data to a computer via USB connection and display the SAF value on the screen.

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| --- | --- | --- |
| Fitzpatrick Class | Characteristics | Cutaneous response to UV |
| I | Bright white                   Northern European/British  | Always burns and never tans  |
| II | White    European/Scandinavian  | Burns easily and tans minimally  |
| III | Fair                                 Southern or Central European | Mild burn and average tanning ability to a light brown  |
| IV | Light Brown                Mediterranean, Asian or Latino | Minimal burn and tans easily to a moderate brown |
| V | Brown                                        East Indian, Native American, Latino or African  | Rarely burn and tans substantially |
| VI | Black                                  African or Aboriginal ancestry | Almost never burns and tans abundantly |

**Table 1** The Fitzpatrick Scale according to skin pigmentation and cutaneous response to UV. Adapted from D’Orazio*et al*.22

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| First Author, year | Subjects (N) | SAF (AU)\* | Reference Group | Follow-up | Outcome |
| De Vos,20144 | PAD (252) | 2.76 | Per unit increase of SAF | 5 yr | Risk for all-cause mortality HR 2.01 and fatal or non-fatal MACE HR 1.82 |
| Meerwaldt,200710 | T1DM (48) T2DM (69) and C(43) | T1DM (0.016) T2DM (0.021) and C (0.010) | Per unit increase of SAF | 5 yr | CHD OR 7,9 and mortality OR 2.0 |
| Gerrits,200811 | T2DM (881) | 2.52 | Per unit increase of SAF | 3 yr | Microvascular complications OR 2.02 |
| Lutgers,200927 | T2DM (967) | 2.69 | Below or above median SAF | 3 yr/ 4yr | All-cause mortality HR 2.05 and CV events HR 1.46 |
| De Vos,201530 | PAD (252) | 2.76 | Below or above median SAF | 5 yr | Amputation sHR 3.05 |

**Table 2** Predictive value of SAF to assess the development of diabetes complications and mortality.

\*SAF values at baseline

AU arbitrary units; CHD coronary heart disease; CV cardiovascular; HR hazard ratio; MACE major adverse cardiac events; OR odds ratio; PAD peripheral artery disease; SAF skin autofluorescence; sHRsubproportional hazard ratio; T1DM Type 1 Diabetes Mellitus; T2DM Type 2 Diabetes Mellitus; yr year.