

# **Simple noninvasive measurement of skin autofluorescence**

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## **Abstract**

Accumulation of advanced glycation endproducts (AGEs) is thought to play a role in the pathogenesis of chronic complications of diabetes mellitus and renal failure. Several studies indicate that tissue AGE accumulation may reflect the cumulative effect of hyperglycaemia and oxidative stress over many years. Simple quantitation of AGE accumulation in tissue could provide a tool for assessing the risk of long-term complications. Because several AGEs exhibit autofluorescence, we developed a noninvasive AutoFluorescence Reader (AFR).

Skin autofluorescence measured with the AFR correlates with collagen-linked fluorescence and specific skin AGE levels from skin biopsies. Furthermore, skin autofluorescence correlates with long-term glycaemic control and renal function, and preliminary results show correlates with the presence of long-term complications in diabetes. The AFR may be useful as a rapid clinical tool for assessment of risk for AGE-related long-term complications in diabetes and in other conditions associated with AGE accumulation.

## **Introduction**

The accumulation of advanced glycation endproducts (AGEs) on tissue proteins has been implicated as a contributing factor in the ageing of proteins and the progression of chronic, age-related diseases, such as atherosclerosis, chronic renal failure, Alzheimer's disease, and diabetes mellitus.<sup>1-8</sup> Glucose initially reacts with proteins to form reversible early glycation products. AGEs develop through complex rearrangements of these glycation products, and decreased renal clearance of these products and/ or oxidative/ carbonyl stress accelerates AGE formation.<sup>1,3,9</sup> The formation and accumulation of AGEs on long-lived proteins affects the structure and function of proteins, enhances cytokine production and activates transcription factors via binding to specific receptors (e.g. RAGE).<sup>10</sup> In diabetes mellitus, AGE accumulation in skin collagen correlates with the duration and severity of hyperglycaemia and with the presence of long-term complications.<sup>6,8,11,12</sup> In a DCCT substudy, skin AGE levels could explain 19-36% of the variance in incidence of long-term diabetic complications in the intensively treated, and 14-51% in the conventionally treated patients. These associations remained demonstrable after adjustment for HbA1c.<sup>8</sup> Even in prepubertal diabetic children AGE levels are increased in relation to glycaemic control and the presence of microvascular complications.<sup>13</sup> Prevention of AGE accumulation experimentally reduces the development of several diabetic complications.<sup>14-16</sup>

The above mentioned studies indicate that tissue AGE accumulation may reflect the cumulative effect of hyperglycaemia and oxidative stress over many years. So in chronic diseases like diabetes, tissue AGE accumulation may represent the existing tissue damage resulting from various known risk factors. Obviously, simple quantitation of AGE accumulation in tissue could provide a tool for assessing tissue injury and the risk of long-term complications.

## **Developing a noninvasive Autofluorescence Reader**

The characteristic fluorescence spectrum of AGEs at 440 nm upon excitation at 370 nm has classically been used to determine tissue AGE accumulation, for example in extracts from homogenates of skin biopsies.<sup>5</sup> Later biochemical and immunochemical assays measure

both fluorescent AGEs, like pentosidine, and non-fluorescent AGEs, like carboxymethyl-lysine (CML). Complexity, cost and lack of reproducibility contribute to limiting broader use of these latter assays. Moreover, blood and urine sampling of AGE do not necessarily reflect tissue AGE levels.<sup>17,18</sup>

Noninvasive techniques to analyze tissue autofluorescence and AGE accumulation have been described by others. For instance, lens autofluorescence is significantly higher for diabetic patients than for age-matched control subjects, and in age-matched diabetic patients the lens autofluorescence increases significantly with the progression of diabetic retinopathy.<sup>19-21</sup> So, the non-invasive evaluation of lens fluorescence is proposed as an early indicator of ocular complications associated with diabetes, and as a screening technique for diabetes. Although in the lens nucleus fluorophores are quite homogeneously concentrated in the centre, other regions of the lens tend to be more variable.<sup>22</sup> Consequently lenses with locally denser cataractous areas, as it frequently appears in cortical cataracts, may cause large measurement variation. Dense cortical opacities may almost totally prevent excitation and thereby emitted light from passing through the lens. The corneal irregularities, eye movements, and corneal AF as well as secondary fluorescence from scattered blue light in the eye tend to obscure *in vivo* measurements.<sup>23</sup> Furthermore, UV radiation may interfere with the assessment of diabetes induced accumulation of lens AGEs.<sup>24</sup>

Several years ago, our clinic analyzed transcapillary and interstitial diffusion of intravenously administered sodium fluorescein as a marker for capillary permeability in diabetes.<sup>25</sup> By chance, we noted increased skin autofluorescence in diabetic patients before the administration of sodium fluorescein, using noninvasive fluorescence capillary microscopy. We developed an instrument, the *AutoFluorescence Reader* (AFR), designed to noninvasively measure skin autofluorescence, because autofluorescence in skin is associated with the presence of AGEs and explains a large part of the variance in the incidence of diabetic complications. We were especially interested in skin autofluorescence, as we preferred to validate this technique against skin biopsy AGE levels in healthy subjects and patients. Furthermore, by measuring at various skin sites (e.g. volar site lower arm, calf, buttock), the results of the AFR might be less influenced by UV radiation, as compared to ocular fluorescence measurements. Finally, the simplicity attracted us to develop a noninvasive skin Autofluorescence Reader.

## The Autofluorescence Reader

Skin autofluorescence is assessed noninvasively by an Autofluorescence Reader (AFR; patent PCT/NL99/00607; prototype of current AGE Reader I, Diagnostica BV, Groningen, The Netherlands). The technique of the AFR has been described in more detail recently.<sup>26</sup> In short, the AFR illuminates a skin surface of  $\sim 1 \text{ cm}^2$ , guarded against surrounding light, with an excitation light source between 300-420 nm (peak excitation  $\sim 350\text{nm}$ ). Care is taken to perform the measurement at a normal skin site, thus without visible vessels, scars, lichenification, or other skin abnormalities. Only light from the skin is measured with a spectrometer (*Ocean Optics* PC-1000 fiber optic spectrometer) in the 300-600 nm range, using 200  $\mu\text{m}$  glass fiber (*Farnell*). The measure of autofluorescence we apply is the average light intensity per nm in the range between 420-600 nm divided by the average light intensity per nm in the range between 300-420 nm (AF in a.u.). We have chosen this ratio to correct for the influence on autofluorescence by light absorption e.g. from skin pigmentation.

Previous results show that repeated AFR measurements on one day show an overall Altman error percentage of <6.0%. Intraindividual seasonal variance shows an Altman error percentage of <6.0%. The differences between repeated measurements do not alter depending on AF level. The AF ratio is calculated off-line by automated analysis and is observer-independent.

## Validation of the Autofluorescence Reader

Recently we validated the AFR against skin biopsy fluorescence and specific skin AGE levels.<sup>26</sup> With the AFR, fluorescence of the skin was measured at the arm in patients with diabetes (both type 1 and 2) and control subjects. Skin biopsies were obtained from the same site of the arm, and analyzed for collagen-linked fluorescence, and specific AGEs: pentosidine, N<sup>ε</sup>-(carboxymethyl) lysine (CML) and N<sup>ε</sup>-(carboxyethyl) lysine (CEL). Skin autofluorescence correlated strongly with collagen-linked fluorescence (CLF), also within each specific diabetic group (table 1.). Furthermore, skin autofluorescence correlated with the specific AGE skin levels of pentosidine, CML, and CEL. Both fluorescent and non-fluorescent skin AGE levels correlated with each other, as indicated by the correlation between pentosidine and CML. Multivariate analysis showed that approximately 50% of the variance in skin autofluorescence could be explained by the independent effects of pentosidine and CML ( $r=0.71$ ,  $p<0.001$ ).

Variables	AF (a.u.)	CLF (a.u./ $\mu$ g hyp.)	Pentosidine (pmol/ $\mu$ g hyp.)	CML (mmol/ mol lysine)	CEL (mmol/ mol lysine)
AF (a.u.)	-	$r=0.62$ **	$r=0.55$ **	$r=0.55$ **	$r=0.47$ **
CLF (a.u./ $\mu$ g hyp.)	$r=0.62$ **	-	$r=0.55$ **	$r=0.50$ **	$r=0.37$ *
Pentosidine (pmol/ $\mu$ g hyp.)	$r=0.55$ **	$r=0.55$ **	-	$r=0.46$ **	$r=0.41$ **
CML (mmol/ mol lysine)	$r=0.55$ **	$r=0.50$ **	$r=0.46$ **	-	$r=0.65$ **
CEL (mmol/ mol lysine)	$r=0.47$ **	$r=0.37$ *	$r=0.41$ **	$r=0.65$ **	-

**Table 1.** Correlation between skin autofluorescence, collagen-linked fluorescence and specific skin AGE levels in validation study for diabetic and control population.

AF= skin autofluorescence, CLF = collagen-linked fluorescence, hyp. = hydroxyproline content of collagen, CML = N<sup>ε</sup>-(carboxymethyl)lysine, CEL = N<sup>ε</sup>-(carboxyethyl)lysine; \* =  $p<0.01$ , \*\* =  $p<0.001$ .

A limitation of the AFR, as a noninvasive measure of tissue AGE accumulation, is that not all AGEs exhibit fluorescent properties. ELISA-based assays have shown increased accumulation of specific AGEs before increased autofluorescence in diabetes.<sup>11</sup> Fluorescence, as expressed in our AF ratio, is a group reactivity, which fails to provide quantitative information on concentrations of individual compounds. Moreover, some non-fluorescent AGEs, like CML have been suggested to have an important pathogenic role. However, interestingly, skin autofluorescence correlated also with skin levels of non-fluorescent AGEs (e.g. CML) in our study. Thus, skin autofluorescence may be a marker of the behavior of a larger pool of skin AGEs, than fluorescent AGE levels alone. Importantly, from a clinical point of view, skin biopsy fluorescence itself is strongly related to long-term diabetic complications.<sup>5,8</sup> Nevertheless, the AFR was only validated against the skin levels of a few specific AGEs. So from a biochemistry point of view, validation of the AFR against other AGE levels deserves attention. This is of great importance if future studies indicate that different AGEs are related to the development of different long-term complications. Studies

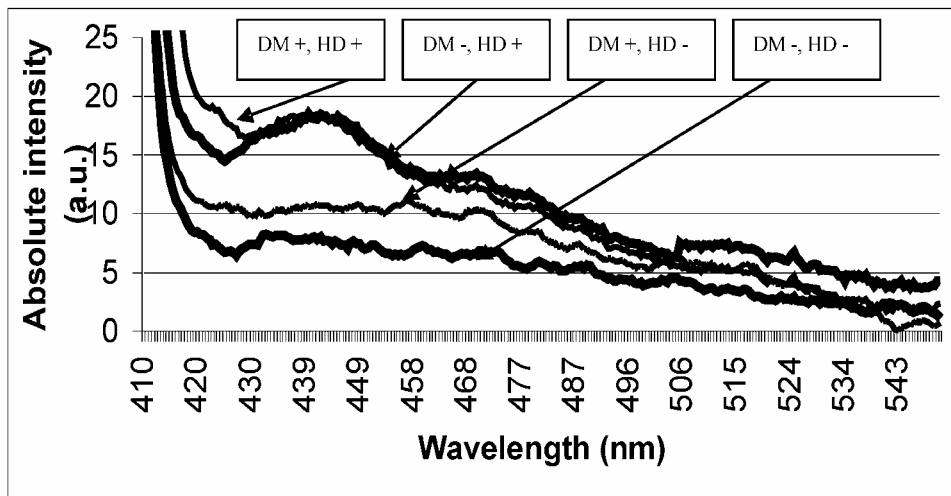
using the AFR with more specific excitation wavelengths are on their way, to further elucidate the characteristic autofluorescence spectra of specific AGEs. This may be clinically relevant to relate certain autofluorescence spectra with the development and progression of different long-term complications in chronic diseases like diabetes.

Sunlight exposure as a source of skin photo aging might be considered as a confounding factor in skin AGE accumulation, but the similar relations we previously found with the AFR for the lower arm and the calf sites suggests that this effect is probably limited. The validation study was performed for the large majority in Caucasian patients and controls and could not address the issue of racial differences. For the moment, our conclusions on the value of skin autofluorescence as a measure of AGE accumulations should be limited to non-pigmented skin, although corrections using differences in skin reflectance may resolve confounding effects of skin pigmentation on skin autofluorescence and seasonal intraindividual variance shows a low Altman error percentage. However, it is important to define the value of the AFR as a measure of AGE accumulation in pigmented skin, as diabetes and cardiovascular disease are seen with a high incidence for instance in Afro-Americans. Further analysis of skin autofluorescence in subjects with pigmented skin are on their way.

So the validation study shows that the AFR measures AGE accumulation noninvasively. Accumulation of AGEs is thought to mediate the deleterious effects of hyperglycaemia and oxidative stress in conditions such as diabetes and renal failure. Consequently, simple and rapid noninvasive measurement of skin autofluorescence holds promise as a convenient tool for assessment of both the risk at related complications and the effects of AGE reduction interventions.

## **The clinical evaluation of skin autofluorescence measurements**

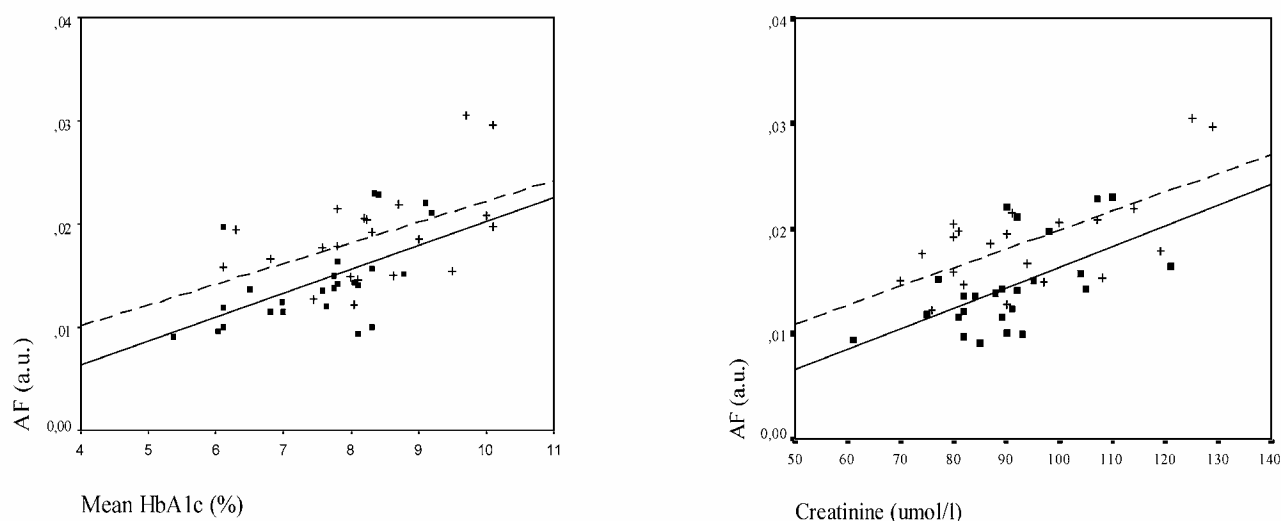
The first clinical evaluation of the AFR in diabetic patients and control subjects showed that skin autofluorescence is increased in diabetic patients (fig 1.) and seems to be related to risk factors for long-term diabetic complications, like ageing and glycaemic control.<sup>26</sup> The strong correlation we found, in both diabetic patients and control subjects, between skin autofluorescence and age is similar to previous observations on the correlation between collagen-linked fluorescence and age.<sup>5</sup> The 2.1 times higher age-related increase in autofluorescence in diabetic patients is also similar to that reported by Monnier for collagen-linked fluorescence. This may be partly explained by the relation we found with diabetes duration, serum creatinine levels and mean HbA1c of the previous year in diabetic patients (fig 2.). Interestingly, skin autofluorescence had a stronger correlation with mean HbA1c of the previous year than with a single HbA1c measurement closest to the study date. So, skin autofluorescence may provide a longer-term index of tissue damage related to metabolic stress, compared to shorter-term measures like glycated haemoglobin.



**Figure 1.** Autofluorescence spectrum in control, diabetic and haemodialysis subjects. DM = diabetic patient, HD = hemodialysis patient.

Preliminary results in a larger group of diabetic patients confirm the above mentioned clinical correlates of age, glycaemic control, and renal function with skin autofluorescence (data submitted). Furthermore, multivariate regression analysis show in this larger group of diabetic patients, that skin autofluorescence is related to the presence of microvascular long-term complications and the presence of coronary heart disease. These clinical correlates remained demonstrable after correction for age and HbA1c. The relevance of the AFR for predicting progression of diabetic complications and mortality is now being investigated in a large (>1000 type 2 diabetic patients), prospective cohort study.

Importantly, skin autofluorescence is not only a measure of glycaemic control, but also correlates strongly with creatinine levels. Renal dysfunction in diabetic patients, but also in euglycaemic patients, contributes to increased tissue AGE accumulation and to the development of cardiovascular complications in these patients.<sup>3,11,12</sup> A pilot study with the AFR in haemodialysis patients showed that skin autofluorescence was severely increased in these patients (fig 1.) and correlated with age, presence of diabetes, duration of renal failure, and duration since the start of dialysis treatment.<sup>27</sup> Mortality rates in patients undergoing haemodialysis are markedly increased, despite measures to improve survival.<sup>1</sup> Cardiovascular disease is the predominant cause of dialysis related mortality. Several risk factors have been recognized and targeted, but mortality rates remain high. Whether skin autofluorescence is related to cardiovascular disease and mortality is now under investigation.



**Figure 2.** Correlation between skin autofluorescence (AF, a.u.) and mean HbA1c (%), and creatinine levels (umol/l) in type 1 diabetic patients (“black box”, continuous line) and type 2 diabetic patients (“cross”, dotted line).

Further analysis of the autofluorescence measurements in diabetic and control subjects show that there is a considerable overlap in skin autofluorescence level between several diabetic patients and control subjects. Interestingly, control subjects with skin autofluorescence levels comparable to some diabetic patients, were all subjects who had smoked for many years. Control subjects who smoked were approximately 15 years older compared to non-smoking, based on their relationship between skin autofluorescence and age. Tobacco smoke contains fluorescent cross-linking proteins, and increases plasma AGEs.<sup>28</sup> In diabetic patients, skin autofluorescence was not related to smoking, which may be explained by the low number of smokers in the diabetic population. Furthermore, the metabolic consequences of diabetes may have a higher impact on AGE accumulation than oxidative stress induced by smoking. Another important exogenous source of AGE accumulation is from nutrition.<sup>29</sup> The results shown by others on AGE accumulation and nutrition, warrant a further analysis of the influence of nutritional AGEs skin autofluorescence.

The existing overlap between diabetic and control subjects and the strong age dependence in autofluorescence limit its use as a diagnostic/ screening test. However, the association of tissue autofluorescence with chronic complications of diabetes, renal failure and other age-related conditions, and experimental evidence for the pathogenic role of AGEs in the development of complications suggest that the AFR offers promise as a tool for risk assessment.

## Conclusion

The AFR offers a simple alternative to invasive measurement of AGE accumulation, sofar validated in non-pigmented skin. Furthermore, skin autofluorescence is related to known risk factors for long-term diabetic complications, like glycaemic control and renal function.

The AFR may be useful as a rapid clinical tool for assessment of risk for AGE-related long-term complications in diabetes and in other conditions associated with AGE accumulation.

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