

## Article: Epidemiology

# Reference values for the Chinese population of skin autofluorescence as a marker of advanced glycation end products accumulated in tissue

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### Abstract

**Aim** Advanced glycation end products play an important role in the pathophysiology of several chronic and age-related diseases, especially diabetes mellitus. Skin autofluorescence is a non-invasive method for assessing levels of tissue advanced glycation end products. This study aims to establish the normal reference value of advanced glycation end products accumulated in tissue measured by the advanced glycation end product reader—skin autofluorescence—and discusses some factors influencing it.

**Methods** The values of autofluorescence in healthy individuals in China were determined by the advanced glycation end product reader; age, gender, skin reflectance, smoking habits and alcohol consumption of the subjects were also recorded.

**Results** The mean reference values of autofluorescence in healthy Chinese subjects are (95% confidence interval) 20–29 years: 1.54–1.62 arbitrary units; 30–39 years: 1.66–1.75; 40–49 years: 1.78–1.89; 50–59 years: 1.87–2.03; 60–69 years: 1.86–2.09; 70–79 years: 1.97–2.31. The value of autofluorescence is strongly related to age, but no significant difference between males and females were found (all  $P > 0.05$ ). Autofluorescence was higher in smokers than in non-smokers ( $P < 0.05$ ). In persons with low skin reflectance ( $< 10\%$ ), skin autofluorescence was dependent on skin colour, but was still related to age.

**Conclusions** The mean reference values of autofluorescence we established could be used for a Chinese population in a clinical setting and are agreement with those in a Caucasian population. Future developments are needed to make the advanced glycation end product reader reliable for lower skin reflections as well, independently of the skin colour.

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**Keywords** advanced glycation end products, advanced glycation end product reader, non-invasive assessment, normal reference value, skin autofluorescence

### Introduction

Advanced glycation end products have originally been described by Maillard, a French chemist, who found that advanced glycation end products were the result of a non-enzymatic chain of chemical reactions between sugars and proteins [1]. Further research revealed that other pathways are also relevant to advanced glycation end product accumulation. Important intermediates in these other pathways are so-called reactive carbonyl compounds, which may form rapidly under oxidative stress by autooxidation of sugars, but also from peroxidation of

fatty acids [2]. Several mechanisms have been proposed by which advanced glycation end products lead to a series of disorders such as: cross-linking with proteins; lipids or nucleic acids causing changes of their structure and function; formation of intracellular advanced glycation end products leading to quenching of nitric oxide and impaired function of growth factors; and binding to receptors such as the receptor of advanced glycation end products on a range of cells leading to activation of key cell signalling pathways with subsequent modulation of gene expression [3]. The role of advanced glycation end products in the pathophysiology of several chronic and age-related diseases has been scientifically well accepted: the advanced glycation end product may play a role in the development of diabetes and its complications [4]. A study of patients with Type 1 diabetes from the Diabetes Control and Complication Trail concluded that skin

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advanced glycation end product levels could explain 19–36% of the variance in incidence of diabetic complications in the intensively treated patients and 14–51% in those who were conventionally treated. The association remained significant after adjustment for HbA<sub>1c</sub>. Tissue advanced glycation end product accumulation as measured from skin biopsies was superior to HbA<sub>1c</sub> measurements in predicting the progression of diabetic complications [5]. From a clinical point of view, it would be desirable to follow up advanced glycation end product accumulation as a consequence of the complex metabolic burden of diabetes in patients with the condition and so offer a predictive measure for the development of long-term complications. However, despite its potential clinical value, tissue advanced glycation end product analysis never reached the clinic, because of the requirement for tissue biopsies. To fit the clinical application, Meerwaldt *et al.* exploited the fact that several advanced glycation end products exhibit autofluorescence and developed a non-invasive tool, the ‘advanced glycation end product reader’, to assess advanced glycation end products accumulated in skin [6].

To validate the advanced glycation end product reader, three separate studies have previously been carried out to compare the non-invasively measured skin autofluorescence value with levels of specific advanced glycation end products measured from skin biopsy homogenates. The first study included patients with diabetes mellitus ( $n = 46$ ) and healthy control subjects ( $n = 46$ ). Skin biopsies were obtained from the same site where the autofluorescence measurements were performed. The study showed that skin autofluorescence correlated strongly with all advanced glycation end products measured from the skin samples, including collagen-linked fluorescence ( $r = 0.62$ ,  $P < 0.001$ ), pentosidine ( $r = 0.55$ ),  $\epsilon$ -carboxymethyllysine ( $r = 0.55$ ,  $P < 0.001$ ) and  $\epsilon$ -carboxyethyllysine ( $r = 0.47$ ) [6]. The second validation study involved 109 patients on haemodialysis treatment and confirmed the same result from the prior study, showing even stronger correlation with collagen-linked fluorescence ( $r = 0.71$ ), pentosidine ( $r = 0.75$ ),  $\epsilon$ -carboxymethyllysine and  $\epsilon$ -carboxyethyllysine (both  $r = 0.45$ ) [7]. A third study was performed in younger persons, again including healthy control subjects, and again showing comparable results [8]. In a combined analysis of these three validation studies, thereby covering a wider age and skin autofluorescence range, the correlation between skin autofluorescence and skin biopsy pentosidine levels rose to 0.87 (A. J. Smit and H. L. Lutgers, unpubl. data).

As skin autofluorescence was associated with both carbohydrate-derived advanced glycation end products (glycoxidation, collagen-linked fluorescence and pentosidine) and lipid-derived advanced glycation end products (lipoxidation,  $\epsilon$ -carboxymethyllysine and  $\epsilon$ -carboxyethyllysine), it can be concluded that skin autofluorescence can be considered as a non-invasive biomarker for chronic cumulative metabolic stress. Since 2004, when the first clinical study on the advanced glycation end product reader was published, more than 40 publications are available. A study by Lutgers *et al.* in 2006

showed some reference values for healthy control subjects using a prototype of the advanced glycation end product reader, but the age range was rather limited and no details were given on factors other than age possibly influencing skin autofluorescence [9]. Previous work showed that skin autofluorescence is affected by skin colour and is significantly associated with skin reflectance ( $R\%$ ) in the lower ranges of  $R\%$  (below 12%) [10]. Recently, the advanced glycation end product reader software has been adapted to extend the range of reliable skin autofluorescence measurements to a lower range of  $R\%$  of 6–8%. Although an article with reference values for Caucasian subjects with ultraviolet (UV) reflection above 10%—as measured with the advanced glycation end product reader—has recently become available [11], the reference values of skin autofluorescence are still unavailable for large cohorts of other populations and skin types. In this study, we will provide the reference values of skin autofluorescence for the Chinese population for different age classes, as well as considering the effect of smoking.

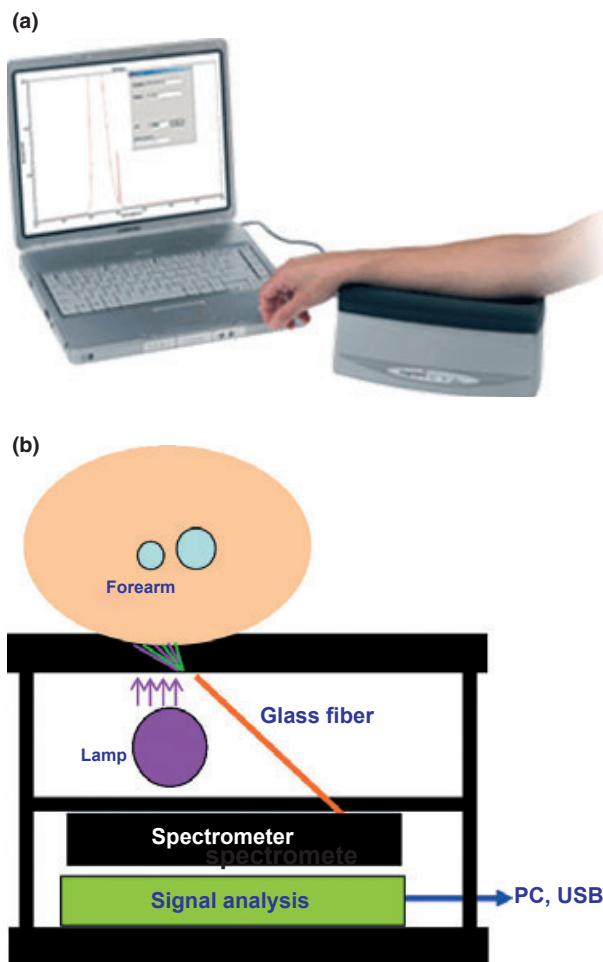
## Subjects and methods

### Subjects

We non-invasively measured skin autofluorescence in 1265 subjects who had a physical examination in our hospital between May and July 2008. Persons without diabetes, hypertension, hyperlipaemia, heart disease, renal dysfunction and any recent infection were selected.

### Methods

Figure 1(a) shows the current advanced glycation end product reader in a clinical setting. According to previous data that show that skin autofluorescence measured at the forearm, lower leg and abdominal area correlated strongly, and because the forearm is the more practical body site for routine measurement, we performed measurements only on the forearm, as in most clinical studies with the device. Figure 1(b) shows a schematic overview of the advanced glycation end product reader, which consists of an UV tube, an optical fibre and a spectrometer. When the measurement is performed, the UV tube emits ultraviolet light (UVA) with a peak wavelength at 370 nm and illuminates the forearm skin of the subject. The light reflected and emitted from the skin in the range of 300–600 nm is transmitted by the fibre probe to the spectrometer. Data from the spectrometer are relayed to a computer, for analysis of the measurement spectrum, and the spectrum and skin autofluorescence value are displayed on the screen. Skin autofluorescence is calculated by dividing the average emitted light intensity per nanometre in the range between 420 and 600 nm by the average reflected excitation light intensity per nanometre in the range between 300 and 420 nm (autofluorescence in arbitrary units). Before the measurement on the skin is performed, two steps have been executed with the shutter closed for calibration: first, reflectance against an internal white reflection standard is measured, which is used for



**FIGURE 1** The advanced glycation end product reader in a clinical setting (a) and a schematic overview of the advanced glycation end product reader (b).

calculation of the skin reflectance value. Second, a dark-current measurement is obtained to correct for the temperature-dependent offset of the charge-coupled device photodetectors, which is subtracted from the spectrum obtained from the white reflection measurement. A dark-current measurement is also performed after the measurement on the subject for correction of the patient measurement. The total measurement procedure is fully automated and takes approximately 30 s. In accordance with the methods described in a prior reproducibility study, where both the day-to-day and seasonal variance showed a mean relative error of 5% [12], we performed all measurements at room temperature (22–24 °C) in a semi-dark environment, and measured the autofluorescence of the skin at the volar side of the arm, approximately 5 cm below the elbow fold. Furthermore, data on skin reflection ( $R\%$ ), gender, age, smoking habits and alcohol consumption were obtained. The local ethics committee approved this study and informed consent was obtained prior to the measurements being taken. The spectra of all measured data have been worked out with the algorithms of software

version 2.2 that are also used by the advanced glycation end product reader, which contains an adaptation for obtaining skin autofluorescence also in the range  $R\%$  7–10%.

### Statistical methods

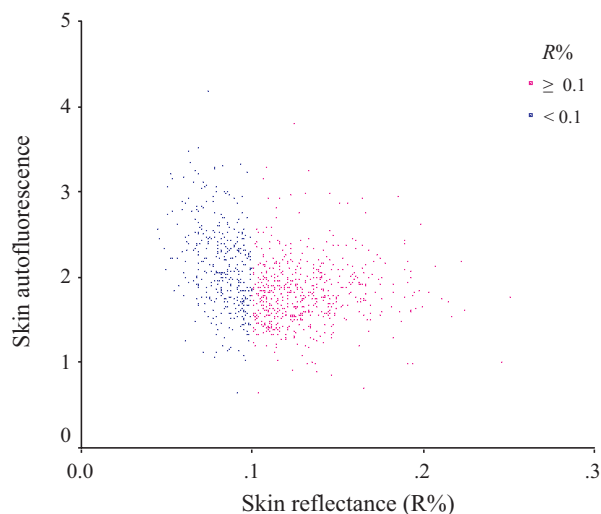
Autofluorescence had a normal distribution (Kolmogorov–Smirnov test,  $P > 0.05$ ). Differences in skin autofluorescence between subgroups (males and females, different age groups, different smoke index groups) were analysed using Student's  $t$ -test. Correlation analyses were performed by Pearson correlation. A two-tailed  $P$ -value of less than 0.05 was considered statistically significant. Data are shown as means  $\pm$  SD.

### Results

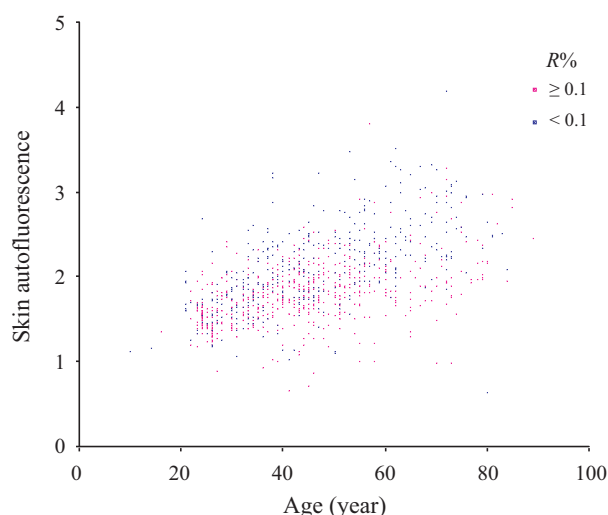
Five hundred and thirty-four females (age  $43.72 \pm 15.0$  years) and 457 males (age  $47.91 \pm 16.31$  years) were included for analysis.

#### Reference values of autofluorescence with influence of reflection, gender and age

Skin reflectance ( $R\%$ ) influences skin autofluorescence. From previous work, it is already known that skin autofluorescence is affected by skin colour and significantly associated with skin reflectance ( $R\%$ ) in the lower ranges of  $R\%$  (below 12%) [10]. In order to determine the threshold of skin reflectance below which skin autofluorescence becomes unreliable, we analysed the relationship between autofluorescence and skin reflectance (Fig. 2), which showed a very steep slope for  $R\% < 10\sim 11\%$ . ANOVA analysis of the relation between autofluorescence and reflectance showed that autofluorescence of different age groups ( $P > 0.05$ ) had no significant variance dependent on  $R\%$ , when



**FIGURE 2** The relationship between autofluorescence and skin reflectance.



**FIGURE 3** The relationship between autofluorescence and age.

$R\% \geq 10\%$ . For the Chinese population,  $R\% = 10\%$  might be the threshold value of skin reflectance for reliable autofluorescence values that are independent of  $R\%$ , using the current advanced glycation end product reader version. It is interesting that, even in subjects with  $R\% < 10\%$ , skin autofluorescence is significantly correlated with age (Fig. 3), suggesting the possibility of improvement of the set-up to be used in subjects with lower skin reflectance.

#### Further analysis of the results with the premise that $R\% \geq 10\%$

As shown in Fig. 3, advanced glycation end product accumulation in human skin tissue is positively correlated with age. To define the best age gradation, we grouped subjects by gender and different age segments, including 5-, 8-, 10-, 13-, 15- and 20-year intervals. The analysis showed that a 10-year interval was the most effective and that no significant differences existed between males and females in each group. The reference values of skin autofluorescence for each age group are shown in

**Table 1** The skin autofluorescence (AF) for each age group

	Age group (years)					
	20–29	30–39	40–49	50–59	60–69	70–79
Male ( <i>n</i> )	34	47	43	26	18	17
Female ( <i>n</i> )	95	73	109	70	26	22
<i>P</i> *	0.09	0.23	0.21	0.2	0.78	0.23
$\overline{AF}$	1.58	1.71	1.83	1.95	1.97	2.14
$SD_{AF}$	0.23	0.26	0.33	0.39	0.38	0.51
95% LL	1.54	1.66	1.78	1.87	1.86	1.97
95% UL	1.62	1.75	1.8	2.03	2.09	2.31

*P*\*, *t*-test of male and female in each age group.  
LL, Lower Limit; UL, Upper Limit.

Table 1. The relation between skin autofluorescence and age can be described in the following formula, which is essentially linear over the complete tested age range: skin autofluorescence =  $1.230 + 0.012 \times \text{age}$  ( $t = 15.726$ ,  $P = 0.000$ ), with age in years. We compared the autofluorescence value observed in the healthy Chinese individuals with values that would have been expected in healthy Caucasian people, calculated from the formula which is given in the article of Koetsier *et al.* [11]. The result shows that there is no significant difference in autofluorescence values between healthy Chinese and Caucasian people in the range from 20 to 60 years of age, as long as the reflection is above the 10% level ( $P = 0.253$ ).

#### Other contributors to skin autofluorescence

##### Autofluorescence and smoking habit

In our study, 178 smokers were selected, ranging in age from 35 to 75 years, and with a smoking dose range from 1 to 90 pack year (pack year = number of cigarette packs smoked per day  $\times$  number of years smoking). In order to address the relationship between skin autofluorescence and smoking, and smoke index, we compared skin autofluorescence values with those of 87 age-matched non-smokers, randomly chosen as control subjects. Taking 0, 10, 15 and 20 pack year as demarcations, we compared the skin autofluorescence of subgroups (subgroup A: smoke index = 0, subgroup B: smoke index < demarcation; subgroup C: smoke index > demarcation) separately. Table 2 shows the results.

The skin autofluorescence of those with > 20 pack year smoking history is significantly higher, not only than that of non-smokers but also than that of subjects with a smoking dose < 20 pack year.

##### Regression analysis

The suspected influencing factors of age, gender and skin reflectance were analysed by regression analysis. The outcome shows that gender has no relationship with skin autofluorescence. When subjects with  $R\% < 10\%$  were included, age and skin reflectance were the influencing factors and explained more than half ( $R^2 = 0.604$ ,  $P = 0.00$ ) of the

**Table 2** The autofluorescence (AF) of subgroups with different smoke index demarcation

			Age	AF	
	Subgroup	<i>n</i>	Mean $\pm$ SD	Mean $\pm$ SD	<i>P</i> <sub>AF</sub>
0 pack year	A	87	49.7 $\pm$ 7.9	1.82 $\pm$ 0.29	0.00
	C <sub>1</sub>	178	50.3 $\pm$ 9.5	2.12 $\pm$ 0.33	
10 pack year	B <sub>2</sub>	29	51.4 $\pm$ 11.3	2.00 $\pm$ 0.19	0.31
	C <sub>2</sub>	149	50.1 $\pm$ 9.3	2.14 $\pm$ 0.35	
15 pack year	B <sub>3</sub>	60	51.0 $\pm$ 9.7	2.03 $\pm$ 0.27	0.51
	C <sub>3</sub>	118	50.0 $\pm$ 9.6	2.15 $\pm$ 0.36	
20 pack year	B <sub>4</sub>	84	49.6 $\pm$ 9.1	1.98 $\pm$ 0.23	0.02
	C <sub>4</sub>	94	50.9 $\pm$ 10.0	2.23 $\pm$ 0.37	

variance in skin autofluorescence in this healthy cohort, with age as the predominant factor ( $R^2 = 0.542$ ,  $P = 0.00$ ). Moreover, when those with  $R\% < 10\%$  were excluded, autofluorescence still correlated with age ( $R^2 = 0.495$ ,  $P = 0.00$ ), independent of the value of skin reflection.

## Discussion

A series of clinical studies on the advanced glycation end product reader showed that skin autofluorescence is not only related with diabetes and its long-term complications, including vascular damage[9], neuropathy[13] [14] and retinopathy[14], but also is an independent and strong predictor of cardiovascular mortality[15], and might be a marker of oxidation stress[16]. Based on the cumulative evidence derived from those studies, we conclude that measuring skin autofluorescence provides an easily applicable, quantitative and reproducible non-invasive tool to investigate the accumulation of advanced glycation end products in larger populations for diagnosis and assessment of the risk of advanced glycation end product-related diseases. Moreover, skin autofluorescence might be a practical tool for estimating risk of complications in diabetes, renal failure and possibly other diseases.

It must be emphasized that, as these results are from a Chinese population, the reference value is strictly applicable only to the Chinese people. The comparison of autofluorescence values observed in healthy Chinese individuals with that which would have been expected in healthy Caucasian people shows that there is no significant difference in autofluorescence values between healthy Chinese and Caucasians in the range from 20 to 60 years of age, as long as the reflection is above the 10% level ( $P = 0.253$ ). However, before this tool is utilized worldwide, its applicability should be verified.

During our study, we came across two problems that must be resolved.

The first problem is the restriction of skin reflection. Our data showed that the current advanced glycation end product reader assesses autofluorescence independent of skin colour, as long as reflectance is 10% or higher, levels of which occurred in 55% of the measured population. The autofluorescence values of those with  $R\% < 10\%$  seem to deviate because of the darker skin colour. However, when  $R\% < 10\%$ , skin autofluorescence is still significantly correlated with age. Improvement of the instrument and regression analysis of larger samples may make it possible for the use of the advanced glycation end product reader to be extended to Chinese subjects with darker skin colour.

The second problem is the restriction of the temperature of the device. As shown previously, the most efficient working time is when the environment is at the correct temperature. According to the working principle of the instrument, we supposed that changes in the characteristics of the spectrometer which are dependent on operating temperature may play a role. It is impossible to prevent the lamp becoming hot, but control over the temperature of the environment, the temperature monitoring

unit and the heat sink would be helpful for an increase in working time. DiagnOptics Technologies (Groningen, The Netherlands) has recently introduced a temperature calibration correction and the results presented in this study have been corrected for the effect of increasing the temperature of the device for longer working times.

Smoking is a source of direct exogenous advanced glycation end products and of induction of oxidative stress. Smoking increases oxygen free radicals in the human body and results in an imbalance of the redox state. As we described previously, the formation of advanced glycation end products is associated with a series of oxidation-reduction reactions. By the use of skin biopsies, it has been proven that advanced glycation end product accumulation in smokers is higher than in non-smokers [17]. Recent results by Koetsier *et al.* show that skin autofluorescence was increased in current smokers compared with non-smoking subjects by an absolute increase of 0.16 arbitrary units and, within the group of current smokers, female subjects had a higher skin autofluorescence than male subjects after correction for age. This suggests that women may suffer more from the effects of smoking than men [11]. Our study concluded that 20 pack year may represent a threshold value above which the effect on skin autofluorescence becomes evident. As smoking is one of the most significant factors to contribute to the accumulation of advanced glycation end products in human body, it is helpful for the clinic to quantify its influence. Autofluorescence is a good indication of the accumulation of advanced glycation end products and we are likely to collect more cases when a conversion factor for smoking need be applied in clinical practice.

By far the greatest contributors of exogenous advanced glycation end products seem to be dairy products, bread and meat, with a strong effect from the way these have been prepared/processed, not only because these foods are all rich in advanced glycation end products, but also as they constitute the bulk of modern food, especially in the Western world. It is also believed that lifestyle leads to variances, as well as some differences between the present results and the results of Koetsier *et al.* in Caucasians [11]. The aspect of lifestyle has not been addressed in the current study.

Last but not least, alcohol is cytotoxic largely because of its metabolite acetaldehyde (AA), the main contributor of advanced glycation end products [18], and abuse of distillate spirits and beer may increase accumulation of advanced glycation end products. In contrast, skin autofluorescence is lower in individuals consuming two or more units of red wine daily [20]. Further analysis on both food and alcohol consumption should be carried out in future research.

In summary, it is concluded that the reference values of Chinese healthy subjects in this study with the advanced glycation end product reader are in agreement with those of Caucasians, at least for subjects with skin reflection  $> 10\%$ , and with an age up to 60 years. For lower reflections, deviations with the current version of the advanced glycation end product reader increase to 30%. Future developments must make the advanced



glycation end product reader reliable for lower skin reflections as well, independently of the skin colour.

## Competing interests

R. Graaff is co-founder and stockholder of DiagnOptics Technologies B.V., the manufacturer of the AGE Reader. M. Koetsier was partly financed by DiagnOptics Technologies B.V.

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