Skin autofluorescence in diabetes mellitus
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Skin autofluorescence for the risk assessment of chronic complications in diabetes: a broad excitation range is sufficient

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ABSTRACT

Introduction Skin autofluorescence (AF) is becoming an accepted clinical method for assessing the risk of chronic complications in diabetes mellitus (DM). In this study, the role of the excitation wavelength in the recognition of increased risk of diabetes-related chronic complications was investigated.

Methods An Excitation Emission Matrix Scanner (EEMS) was used to perform non-invasive measurements in four age-matched groups of patients with type 1 and type 2 DM, with and without chronic complications, as well as in a control group (N=97 in total). AF was calculated for excitation wavelengths in the range 355 - 405 nm. Mean spectra were assessed per group.

Results AF values in both type 1 and type 2 DM patients with complications were increased compared to the control subjects (p<0.01); this ratio remained practically constant, independent of the excitation wavelength. No emission peaks were distinctive for specific patient groups.

Conclusion We conclude that in these groups, no characteristic fluorophores dictate the use of a specific wavelength or set of wavelengths. The results show the validity of applying a broad excitation wavelength range for risk assessment of chronic complications in diabetes.

INTRODUCTION

Autofluorescence of human tissue upon excitation with UV-A light, is caused by endogenous fluorophores including collagen, elastin, NADH, tryptophan and porphyrins [1,2]. Application of tissue autofluorescence in vivo has been used previously to detect local disorders such as cancer [3-8], age-related macular degeneration [9], psoriasis [10], and skin burns [11] and the effects of chronic ultra violet exposure [12].

Moreover, determination of skin autofluorescence from biopsies has been used in studies on systemic diseases such as diabetes mellitus (DM) [13]. Advanced glycation endproducts (AGEs) that cross-link collagen, are the main source of collagen linked
Skin autofluorescence and excitation-emission maps

fluorescence (CLF) in skin biopsies and are classically determined by fluorescence at 440 nm upon excitation at 370 nm [13]. AGEs are products of glycemic and oxidative stress, and their formation is increased in pathological conditions like DM, renal disease, and also in atherosclerosis. [14-18] Tissue-AGE determination is usually performed in skin biopsies.

Several investigators have studied skin or lens autofluorescence non-invasively [19-23]. We developed an Autofluorescence Reader (AFR) for non-invasive measurement of skin autofluorescence [24-27]. With this AFR, a prototype of the current AGE Reader (DiagnOptics, The Netherlands), the skin on the volar side of the forearm is illuminated with light in an excitation range of 350 - 420 nm (maximum intensity at 370 nm), and emission in the range 420 - 600 nm is measured with a spectrometer. A strong correlation was not only found with CLF, but also with skin AGEs (pentosidine, N\textsuperscript{ε}-carboxymethyllysine, N\textsuperscript{ε}-carboxyethyllysine). These skin AGEs were assessed in dermal tissue of skin biopsies from DM patients, renal failure patients and healthy persons, taken from the same site as where the autofluorescence measurements were taken [24,28]. In later studies, AF has been studied in a range of other conditions such as atherosclerosis, preeclampsia, systemic lupus erythematosus (SLE), and glycogen storage disease (GSD) Ia [29-32]. Moreover, AF has been reported to be an independent predictor of macrovascular and microvascular complications [33] and of mortality in hemodialysis and DM patients [28,34].

The spectrum of the UV-A light source as used in the AFR and in the AGE Reader is shown in Figure 1 (continuous line). With the broad and fixed excitation range of this setup, we were unable to further identify specific fluorophores or AGEs. Therefore, an instrument was developed with a similar setup as in the AFR, but where excitation wavelengths could be varied: the Excitation-Emission Matrix Scanner (EEMS), as previously described by Graaff et al [25]. With this equipment, matrices of the amount of fluorescence as a function of excitation and emission wavelengths can be obtained from the skin in vivo to further investigate the skin fluorophores that are involved in various clinical conditions. This study will concentrate on EEMS measurements obtained from DM patients with and without DM-related chronic complications.
The aim of this study is first to determine which excitation and emission wavelengths are optimal for differentiation between diabetic and non-diabetic subjects or between diabetic subjects with and without DM-related chronic complications. And secondly, to evaluate whether the presence of DM-related chronic complications is associated with differences in fluorescence spectra.

METHODS

Subjects Excitation-emission spectra were collected in groups of patients with type 1 and type 2 diabetes, with and without chronic complications, as well as in a control group, see Table 1. Diabetes patients were recruited from the University Medical Center Groningen. All participants gave informed consent to this study which was approved by the local medical ethics committee.

All participants had an age between 35 and 50 years. This limited range of age was chosen, since the amount of accumulated AGES increases with age [13,27]. Only Caucasian patients were included in this study, because dark skin may influence AF assessment [26]. We included 17-24 subjects in each group. Patients were classified as having chronic complications when retinopathy, neuropathy, microalbuminuria or macrovascular disease were present, all defined according to definitions described in detail previously [27,33]. Table 1 shows the characteristics of the five subject groups.

<table>
<thead>
<tr>
<th></th>
<th>DM type 1 without compl.</th>
<th>DM type 1 with compl.</th>
<th>DM type 2 without compl.</th>
<th>DM type 2 with compl.</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>19</td>
<td>17</td>
<td>21</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
<td>42.3 ± 4.5</td>
<td>43.2 ± 4.6</td>
<td>45.8 ± 4.7</td>
<td>47.5 ± 5.1</td>
<td>46.8 ± 1</td>
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<tr>
<td><strong>Gender (M:F)</strong></td>
<td>8:11</td>
<td>6:11</td>
<td>15:6</td>
<td>10:6</td>
<td>7:17</td>
</tr>
<tr>
<td><strong>Diabetes duration (yr)</strong></td>
<td>20 ± 11</td>
<td>28 ± 7</td>
<td>5 ± 1</td>
<td>9 ± 6</td>
<td>-</td>
</tr>
</tbody>
</table>
Equipment and Measurements For generating excitation dependent emission spectra, an instrument had been developed where excitation wavelengths can be varied. This instrument was obtained by adaptation of a 0.2 m f/4 monochromator (PTI, New Jersey, USA), and was illuminated by a 75 W Xenon lamp. The monochromator has been connected to a measuring section, where a glass fiber (with a diameter of 200 µm) is pointed at the measuring site under an angle of 45 degrees. This glass fiber is connected to a spectrometer (USB2000, Avantes, Eerbeek, The Netherlands). The total setup, referred to as Excitation Emission Matrix Scanner (EEMS) has a similar setup as the previously described tool for measuring skin autofluorescence, the AFR [25]. Wavelength scanning of the monochromator was realized with a PC using a LabView program (National Instruments, Austin, USA). The spectra were obtained by this software as well. Measurements were performed on the volar side of the arm, approximately 10 cm below the elbow. A whole series of measurements, including measurements at 11 excitation wavelengths (355 - 405 nm, in steps of 5 nm) was performed in a few minutes. Optimal integration times were determined by the LabView program, therefore, all spectra were measured in the same dynamic range of the spectrometer. Dark spectra were also obtained and subtracted for all applied integration times. The shape of the peak at each selected excitation wavelength can be described by a normal distribution with a standard deviation of 6.5.

Analyses and statistics Autofluorescence as obtained from the EEMS was defined as a function of excitation wavelength for all subjects as

$$\text{AFE}(\lambda_{\text{exc}}) = 100 \times \frac{<I_{\text{em}} >}{<I_{\text{exc}} >}$$  \hspace{1cm} equation (1)

where $<I_{\text{em}} >$ is the mean measured light intensity in the range 420 - 600 nm and $<I_{\text{exc}} >$ is the mean intensity in the range 300 - 420 nm, both for excitation wavelength $\lambda_{\text{exc}}$. This same method is used in the AGE Reader to calculate AF from the spectrum. After calculating AFE for all subjects, the mean AFE for subjects in the control group per excitation wavelength was used as a reference for all four DM subject groups.
For comparison between AF values of DM subject groups and the control group, we introduced relative autofluorescence as

$$AF_{E,rel}(\lambda_{exc}) = \frac{<AFE(\lambda_{exc})>_{patient}}{<AFE(\lambda_{exc})>_{control}}$$

*equation (2)*

where $<AFE(\lambda_{exc})>_{patient}$ is the mean $AFE(\lambda_{exc})$ of a DM subject group and $<AFE(\lambda_{exc})>_{control}$ is the mean $AFE(\lambda_{exc})$ of the control group. For each excitation wavelength and for all DM groups separately, a Mann-Whitney U-test was performed against the control group to evaluate the significance of the difference. These tests were performed using SPSS.

In the AGE Reader, a blacklight is used, emitting a broad band of UV light, as shown in *Figure 1*. In order to derive an analysis for this light source, a weighted summation of measured responses on excitation at given wavelengths was used. The AF resulting from the blacklight, $AF_{BL}$, per subject was calculated using

$$AF_{BL} = \frac{\sum w_i \ AF_i}{\sum w_i}$$

*equation (3)*

where $AF_i$ is $AFE$ as calculated from excitation peak $i$ and the weight factor $w_i$ was determined by multiple regression fitting of the separate peaks to the broad excitation peak. The broad excitation peak that can be reconstructed in this way is shown in *Figure 1* (dotted line). The Mann-Whitney U-test calculations were also performed for $AF_{BL}$.

![Figure 1](image.png)

*Figure 1*. Spectrum of the UV-A light source as used in the AFR and the AGE Reader (continuous line) and a fit using a weighted summation of EEMS responses of separate excitation peaks (dotted line). The small peaks at 366 nm and at 405 nm are caused by mercury emission within the lamp.
To analyze possible differences in the emission spectra between the groups, the spectra were all normalized. The mean value of intensity of the emission peak (in the range 450 - 600 nm) was taken as a reference. Subsequently, mean values were calculated (per nanometer) from the normalized spectra of all subjects in each group for each excitation wavelength. These mean spectra were used to observe whether emission peaks occurred that might be specific for a certain patient group.

RESULTS

Autofluorescence ratio

Autofluorescence values ($AF_E(\lambda_{ex})$) were obtained for all subjects for excitation wavelengths in the range 355 - 405 nm in steps of 5 nm. Figure 2A shows the mean relative $AF_E(\lambda_{ex})$ values for the four groups. The standard deviation of the AF values within each group ranged between 18% and 36% of the mean value. Instead of error bars, Figure 2B shows the significance of the differences between groups. The figure shows a significantly increased mean $AF_E$ for the two groups of DM subjects with chronic complications ($p < 0.01$) for all excitation wavelengths. Also the group of type 1 DM subjects without chronic complications tends towards an increased AF. It should be noted that the measurements with an excitation wavelength of 360 nm were discarded from this part of the study, because of technical problems during the measurements. Finally, equation (3) was used to calculate results for the broad excitation peak of the AGE Reader. Figure 2 shows similar results for the broad excitation peak as compared to the separate narrow excitation peaks. No significant differences in mean autofluorescence values between subjects within each group were found for smoking, gender and age. The difference between the groups with DM type 1 and type 2 patients without complications that can be seen in Figure 2B is not significant ($p > 0.05$) and may be caused by the longer diabetes duration of the DM type 1 patients.
Figure 2. A. Mean relative autofluorescence for the separate excitation peaks \( AF_{E,rel}(\lambda_{ex}) \) and the broad excitation peak from the blacklight (BL) as used in the AGE Reader. B. Statistical significance (Mann-Whitney U-test) of the differences between \( AF_{E} \) values of subjects in the respective subject groups and the control group. Squares denote the groups of subjects with type 1 DM, diamonds denote type 2 DM. Closed figures denote the groups of subjects with chronic complications, open figures denote the groups of subjects without complications.

Figure 3. Emission peaks from the average spectra of the control group for increasing excitation wavelength. The spectra are normalized for emission.

Normalized spectra. The emission peaks of the normalized spectra for the control group are displayed in Figure 3. The shape of the spectrum is slightly changing for different excitation wavelengths. However, the maximum intensity remains at approximately 500 nm, independent of excitation wavelength. The normalized spectra
of the four patient groups have a similar shape. The emission spectra of all five groups are shown in Figure 4 for excitation wavelengths of 375 nm (a) and 400 nm (b). These figures clearly show the similarity of the emission spectra between groups. The examples are typical for all other excitation wavelengths as well.

![Figure 4](image)

**Figure 4.** Emission peaks from the average spectra of the five groups for $\lambda_{\text{exc}} = 375$ nm (a) and $\lambda_{\text{exc}} = 400$ nm (b). In (b), the spectrum starts at 443 nm as below that wavelength, the excitation peak is still present.

**DISCUSSION**

No specific excitation or emission wavelengths were found that would yield an increased distinction between the groups of patients with both types of DM, with or without chronic complications, or the control group. In fact, our results show almost constant factors and significances for all excitation wavelengths. Consequently, about the same factors occur for the broad excitation band from the blacklight as used in the AGE Reader. From the observation that the emission spectra of the five groups are very similar for each separate excitation wavelength, it can be concluded that no fluorophores dictate the use of specific excitation or emission wavelengths in these
patient groups. Therefore, the shapes of the spectra do not add more information to skin autofluorescence as determined by the AGE Reader for recognizing DM or chronic complications in DM. Moreover, from our results, all fluorophores seem to be equally increased in the groups of patients with complications.

The observed broad emission spectrum as seen in the normalized spectra could be expected, since many fluorophores exist in the skin [22,35]. It should be noted that variation of the excitation wavelength only yields a few changes in the shape of the emission spectrum. This implies that the different skin fluorophores have at least overlapping excitation spectra. Our results suggest that the role of excitation wavelength may not be very important to detect clinical differences, a suggestion that also raised in the field of cancer detection [36].

It is a remarkable observation that the shapes of the emission spectra for a given excitation wavelength are similar for the different groups, even as compared to the controls. The high number of different fluorophores in the skin and their interwoven emission spectra as well as the turbid nature of tissue, make it very difficult to recognize specific fluorophores that might cause differences between the groups. Furthermore, the spectra may be influenced by the location of the fluorophores in the skin, the biochemical and biophysical environment, and the presence of other (non-fluorescing) chromophores [22,37,2]. We speculate that the same pool of fluorophores causes fluorescence in healthy subjects as in DM patients, since only the amount is different, not the composition.

Our initial expectation was that we might see specific AGE-related emission peaks in metabolic diseases like DM as compared to control subjects, since AGEs have an essential role in the development of chronic complications in DM [13,38,39]. We furthermore expected a possible difference between type 1 and type 2 DM, as these diseases with different metabolic conditions could generate different AGEs. Collagen-linked 370/440 nm fluorescence, as a marker of AGE accumulation, was a proven predictor of these complications in earlier studies on skin biopsies, and fluorescent AGEs such as pentosidine and argpyrimidine are also related to such conditions. These and some other fluorescent compounds (for example NADH) might result in clinically
relevant specificities in excitation-emission pairs. However, attempts to derive specific emission peaks from our measurements between different groups have not yet been successful; the clinical differences manifest themselves proportionally for all excitation-emission pairs.

Even without an exact knowledge of the composition of the fluorophores, a correlation exists between the amount of total autofluorescence and presence of DM-related chronic complications, as has been reported before [27,34]. The study of Lutgers et al reported an increased AF for type 2 DM patients without chronic complications, while our study showed no increased AF in this group [27]. The type 2 DM population in that study was however a large unselected cohort. Our rather small group of type 2 DM patients without complications was specifically selected for having type 2 DM and being in perfect health otherwise. However, our results show a clear distinction of the groups of subjects with chronic complications in either DM type, with highly significant differences compared to controls and almost constant factors for all excitation wavelengths. These significant differences remain present for a broad excitation band from a blacklight as used in the AGE Reader. This finding confirms the validity of using a broad excitation wavelength for distinguishing complications in conditions such as diabetes mellitus.

CONCLUSION
Our results show that skin autofluorescence at all excitation wavelengths in the range 355 - 405 nm equally distinguishes increased risk of DM-related chronic complications in Caucasian subjects. The fluorophores do not dictate the use of a specific wavelength or set of wavelengths in assessing this risk. These results therefore show the validity of a broad excitation wavelength range, such as applied in the AGE Reader.

REFERENCES
Chapter 2


