Improving the measurement of skin autofluorescence, or working on the clinical applicability of skin autofluorescence from a biomedical engineering perspective

Marten Koetsier

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Stellingen

behorende bij het proefschift

Improving the measurement of skin autofluorescence, or working on the clinical applicability of skin autofluorescence from a biomedical engineering perspective

1.
Met een lichtbron met een breed excitatiespectrum kan men een cardiovasculair risicoprofiel vaststellen.
(dit proefschrift)

2.
De AGE Reader is geschikt voor het bepalen van de humane biologische leeftijd.
(dit proefschrift)

3.
Vrouwen ondervinden meer schade door roken dan mannen.
(dit proefschrift)

4.
De invloed van de huidskleur op de gemeten autofluorescentiewaarde van de huid is afhankelijk van de leeftijd.
(dit proefschrift)

5.
De huid is de spiegel van het lichaam.
(dit proefschrift)

6.
Ook zonder van de hoed en de rand te weten kan men wetenschap bedrijven.

7.
Discrimineren op basis van huidskleur is een zeer oppervlakkige bezigheid.

8.
Kleurenblindheid geeft het leven meer kleur.

9.
De Groningse overheid slaagt er niet in het fietsen in de stad een halt toe te roepen.

10.
Zowel privatisering als de verregaande schending van de privacy ondermijnen de samenleving.

11.
Universiteiten en ziekenhuizen moeten voorop lopen in gebruik van open bestandsformaten.

12.
In de wetenschap zijn cultuurverschillen tussen verschillende disciplines vaak groter dan cultuurverschillen tussen mensen uit verschillende delen van de wereld.

13.
Hofstadter's Law: It always takes longer than you expect, even when you take into account Hofstadter's Law.
(Douglas Hofstadter, Gödel Escher Bach: an Eternal Golden Braid.)

Marten Koetsier
23 maart 2011

Koetsier, M

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„Alles leeft, kleine jongen," sprak zij, ,,men moet het alleen weten te zien".

Godfried Bomans – „Erik of het klein Insectenboek" – 1941

Contents

List of abbreviations

AF	autofluorescence
AFR	autofluorescence reader
AGE	advanced glycation end product
AU	arbitrary units
BL	blacklight
CEL	N^{ϵ}-(carboxyethyl)lysine
CIE	Commission Internationale de l'Éclairage (International Commission on Illumination)
CLF	collagen linked autofluorescence
CML	N^{ϵ}-(carboxymethyl)lysine
DFN	Diabetes Fonds Nederland (Dutch Diabetes Fund)
DM	diabetes mellitus
EASD	European Association for the Study of Diabetes
EEM	excitation emission matrix
EEMS	EEM scanner
ESRD	end-stage renal disease
FAD	flavin adenine dinucleotide

FLIM	fluorescence lifetime imaging
FWHM	full width at half maximum
GSD	glycogen storage disease
LED	light emitting diode
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
PC	personal computer
PF	plasma fluorescence
SLE	systemic lupus erythematosus
STEMI	ST-elevation myocardial infarction
TRFS	time-resolved fluorescence spectroscopy
UKPDS	United Kingdom prospective diabetes study
WHO	World Health Organisation

CHAPTER 1

Introduction

Autofluorescence of human skin (skin AF) has been shown to be an independent predictor for development and progression of complications in diabetes mellitus (DM), renal failure and other diseases with increased cardiovascular risk.[1–3] A significant correlation exists between skin AF and skin levels of advanced glycation end products (AGEs) like pentosidine, N^{ϵ}-(carboxymethyl)lysine (CML) and N^{ϵ}-(carboxyethyl)-lysine (CEL), as obtained from skin biopsies.[1, 4, 5] These AGEs accumulate over time in tissue and the rate of accumulation is increased in patients with the conditions mentioned above. Moreover, AGEs contribute to the development of medical complications, especially cardiovascular.[6] The fluorescent properties of some of the large pool of AGEs facilitate the assessment of cardiovascular risk by measurement of skin AF. Skin AF can be measured with the AGE Reader, a device developed by the Departments of Biomedical Engineering and Vascular Medicine of the University Medical Center in Groningen, and further industrially developed and commercialized by DiagnOptics Technologies B.V., a university spin-off company that was founded for that aim.

AGEs and fluorescence

The working principle of the AGE Reader relies on the measurement of fluorescence. A molecule that has the property of fluorescence is able to absorb a photon of a certain wavelength or energy. The amount of energy, E, relates to the wavelength, λ, via the Planck-Einstein equation

$$E = \frac{hc}{\lambda} \tag{1.1}$$

where h is Planck's constant (6.626×10^{-34} Js) and c and λ are the speed and wavelength of light in the medium. The energy is used to push the fluorophore into a higher quantum state. This excited electron will fall back into its original quantum state, releasing energy again in the form of a photon. However, part of the energy is dissipated as heat, and the photon that is emitted has a lower energy and therefore a higher wavelength. A schematic overview of this process is plotted in Figure 1.1.

Each fluorophore has one or more wavelengths at which excitation is optimal. At wavelengths close to this maximum, excitation also occurs, but to a lesser extent. A fluorophore can thus be characterized by its excitation spectrum. The fluorophores as present in the skin that have an excitation maximum in the 350 – 410 nm range are listed in Table 1.1, which was derived from a review of Bachmann et al.[7] Many other fluorophores have an optimal excitation wavelength outside this range, but may also

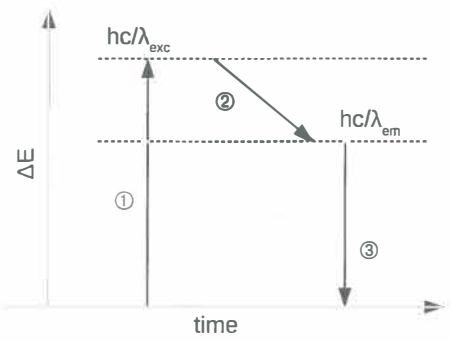

Figure 1.1: *Schematic drawing of the fluorescence process in time. The vertical scale represents the energy needed to excite a molecule. ① A photon of energy hc/λ_{exc} excites the molecule, pushing the fluorophore into a higher quantum state. ② Part of this energy is dissipated as heat. ③ When the electron relaxes into its original quantum state, a photon of lower energy hc/λ_{em}, and therefore higher wavelength, is emitted.*

Table 1.1: *Some fluorophores in the skin with excitation maxima in the 350 – 410 nm range and their emission maxima.*

Fluorophore	maximum excitation (nm)	maximum emission (nm)
Collagen crosslinks	335, 370	390, 460
Lipofuscin	340 – 395	540, 430 – 460
Ceroid	340 – 395	430 – 460, 540
NADH	350	460
Keratin	370	460
Vitamin D	390	480
Porphyrins	400 – 405	600, 630

contribute to the overall excitation of the skin because they can still be excited within this range. The AGE pentosidine is one of these compounds, having an excitation optimum around 330 nm.[8]

Due to the overlapping nature of excitation and emission spectra, it is difficult, if not impossible, to distinguish specific fluorophores such as AGEs from in vivo measurements. Moreover, only a part of the many different AGEs have fluorescent properties. However, it has been shown that the dermal content of specific AGEs, including non-fluorescent AGEs, explains the major part of the variance (up to 76%) in the skin *AF* signal.[9] Therefore, the measurement of skin autofluorescence provides a good estimate of dermal AGE content.

Dermal skin tissue is an example of a tissue with a slow turn-over. It was estimated that dermal skin collagen has a lifetime of 15 years.[10] Because the lifetime of AGEs is critically dependent on the proteins to which they are irreversibly cross-linked, skin *AF* thereby provides an estimate of the long-term accumulation of AGEs on proteins with a slow turn-over in other tissues. Because the effects of an AGE are probably more determined by the accumulation- and time-dependent degree of modification of proteins than by the specific type of AGE, assessment of AGEs in tissue with slow turn-over allows to better estimate the effects of AGEs than in tissues with rapid turn-over such as blood or mucosa.

Development of the AGE Reader

The development of skin autofluorescence measurements started after the first notion of increased background fluorescence in non-invasive in vivo video microscopy of capillary sodium fluorescein leakage in DM patients.[11] In the 1980's it was already known that collagen linked autofluorescence (CLF) in skin biopsies is correlated with complications in DM.[12] However, the measurement of CLF requires a biopsy and a substantial specimen preparation and lab assessment and therefore, this invasive technique can not easily be used. Jager et al. were the first to non-invasively measure increased autofluorescence in DM patients.

The first proof of principle was presented at the 35th meeting of the European Association for the Study of Diabetes (EASD) in 1999.[13] The setup needed for this study was developed at the Department of Biomedical Engineering of the University of Groningen and was referred to as autofluorescence reader (AFR). It consisted of a white box of approximately 35 cm long, containing an 8 W UV blacklight tube and a fixture for a 200 μm optical fiber, leading to an external spectrometer, see Figure 1.2.

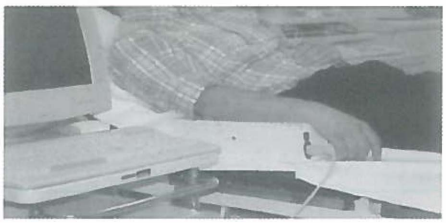

Figure 1.2: *Prototype AFR as used by Meerwaldt et al. in 2005. This setup consisted of a white box with the UV lamp as measuring site, with an external spectrometer connected to a PC.*

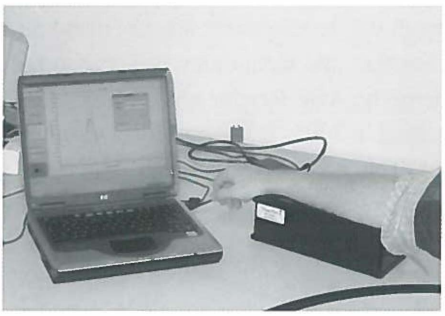

Figure 1.3: *The first table-top prototype of the AGE Reader in 2005 had all functional components embedded, and a PC was used to evaluate the measurement and calculate skin autofluorescence.*

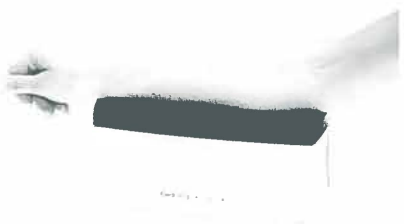

Figure 1.4: *The current AGE Reader as a validated device with CE certification since 2006.*

An aperture in the housing was used as a measuring window. The spectrometer was an expansion card in a personal computer, equipped with software to obtain and store the spectra. The light source emitted light in the 300 – 420 nm range with a peak intensity around 350 nm. Skin AF was calculated as

$$AF = \frac{I_{em}}{I_{exc}} \tag{1.2}$$

where I_{em} is the the average light intensity as measured in the 420 – 600 nm range, and I_{exc} is the average intensity in the 300 – 420 nm range. In a later version, a different light source was used with a peak wavelength around 370 nm. Also a more advanced, external spectrometer was used, connected with a 50 μm optical fiber. With the latter setup many clinical validation studies have been performed.

The next major step in the development was to embed all functional parts of the setup in one device. For this, the technology was transfered to DiagnOptics. The first table-top prototype of the AGE Reader was developed by this company and was presented at a SPIE meeting in 2005.[14] This industrial prototype consisted of a box of approximately 25×10×10 cm and housed the light source, the spectrometer, a shutter with in-built white reference standard, and electronics for automated operation. It was connected to a personal computer via a USB connection. A software program was developed to completely automate the measurement, including dark measurements used for subtracting dark current in the spectrometer. A picture of the first automated prototype of the AGE Reader is provided in Figure 1.3. DiagnOptics later introduced the AGE Reader, see Figure 1.4, and acquired CE certification in 2006.

The working principle of the AGE Reader is as follows. A blacklight tube, with a peak wavelength of 370 nm is used to illuminate ~4 cm^2 of the skin on the volar side of the forearm. A sample spectrum of this light source is shown in Figure 1.5. A non-contact optical fiber detects the emission and reflected excitation light at an angle of 45°. Using a spectrometer and computer software, the intensity spectrum is analyzed. The value of skin AF is still being calculated using Equation 1.2, now multiplied by 100 to yield manageable values, and is expressed in arbitrary units. Besides the skin AF measurement, a diffuse reflection spectrum in the excitation range is obtained by normalizing the measured spectrum to a spectrum as measured on a white reflection standard. Additionally, to obtain an optional reflection spectrum in the visible range, a white light emitting diode (LED) was added as illumination source. Information on the diffuse reflection in the visible part of the spectrum may provide additional information, e.g. about skin color, that can be used to improve the accuracy and interpretation of skin AF measurements. The fluorescence and reflectance spectra are

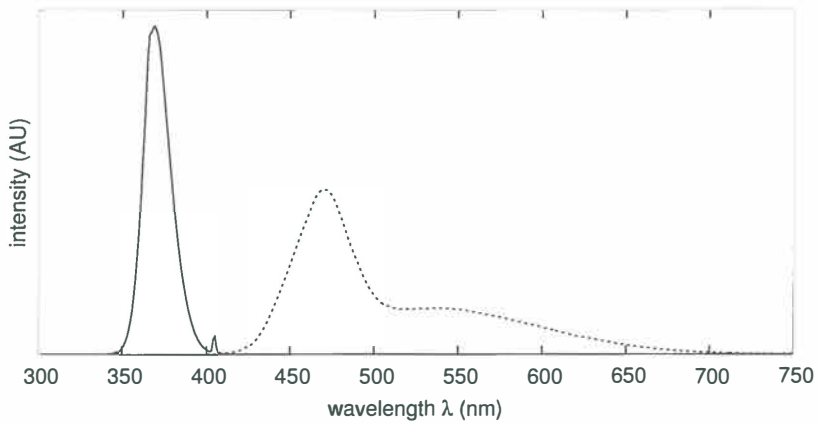

Figure 1.5: *Sample spectrum of the two light sources used in the AGE Reader. The solid line represents the UV-tube, the dashed line represents the white LED. The spectra are scaled and plotted in arbitrary units (AU).*

corrected for dark current. All spectra, including the dark current spectra, are stored in a file for later analysis. The skin *AF* value and UV reflectance are immediately calculated, stored and displayed by dedicated PC software.

Clinical application

The first validation studies for the AFR showed a good correlation between skin *AF* and tissue levels of pentosidine, CML and CEL in DM patients and age-matched healthy controls[4] and in patients suffering from end stage renal failure.[1] In a pooled analysis, also including a study by den Hollander et al. in younger healthy controls,[5] tissue levels of pentosidine from dermal biopsies could explain 76% of the variation in skin *AF* values measured at the same site with the AFR.[9]

In a large group of DM patients (n = 973), it was shown that skin *AF* was higher in patients with type 2 DM than in healthy control subjects and that it was associated with a graded increase in the presence and severity of diabetes-related complications.[15] Skin *AF* also showed a correlation with nerve conduction velocities as manifestation of diabetic neuropathy, even in the preclinical stage.[16] In a follow-up study, the (independent) predictive value of skin *AF* for macrovascular and microvascular complications was unequivocally demonstrated.[3, 17] Moreover, it was

shown that skin *AF* provides additional information to existing risk analysis methods, in this case the United Kingdom Prospective Diabetes Study (UKPDS) risk engine. This resulted in risk-reclassification of a substantial number of patients and identifying patients with a particularly high risk for developing cardiovascular events.[3] It was also shown that skin *AF* is an independent predictor for the development of microvascular complications in type 2 DM.[17]

Skin *AF* has also shown to be increased in dialysis patients.[1, 18] Even within a cohort of dialysis patients, a higher mortality rate was shown in patients with higher skin *AF*.[1] Diastolic dysfunction in dialysis patients, as assessed using tissue velocity imaging, was related to an increase in skin *AF* as well.[19] In renal transplant recipients, skin *AF* was associated with several risk factors for cardiovascular disease and chronic renal transplant dysfunction.[20] Skin *AF* was even found to be an independent predictor of graft loss and mortality in kidney transplant recipients.[21]

A correlation was found between skin *AF* and carotid intima media thickness,[22, 23] also in patients suffering from systemic lupus erythematosus (SLE).[24] Furthermore, skin *AF* adds incremental information to global risk assessment scores in identifying asymptomatic subjects at high risk for cardiovascular disease.[22] It was also shown that skin *AF* is elevated in stable coronary artery disease and that it is related to serum levels of the soluble receptor for AGEs and neopterin.[2] Skin *AF* is also elevated in patients with ST-elevation myocardial infarction (STEMI), is associated with inflammation and glycemic stress, and predicts future major adverse cardiac events in STEMI patients.[25] Finally, skin *AF* has also been found to be increased in other conditions like preeclampsia[26] and autoimmune disease (SLE and Wegener's granulomatosis),[24, 27] which are also associated with increased cardiovascular risk.

Nowadays, the AGE Reader is increasingly used worldwide for studying the role of AGEs and clinically for cardiovascular risk prediction. Skin *AF* was related to hyperglycemia, adiposity and metabolic syndrome in an Italian type 2 DM population.[28] In Japan, skin *AF* was related to oxidative stress in DM and renal failure, as well as in chronic cerebral ischemia,[29] to rheumatoid arthritis and dialysis-related spondyloarthropathy[30] and to renal function and cardiovascular diseases in pre-dialysis chronic kidney disease patients.[31] Furthermore, skin *AF* was shown to correlate with arterial stiffness in end-stage renal disease patients and healthy subjects.[32] A French study showed that skin *AF* is associated with nephropathy in DM type 1 patients.[33] In the United Kingdom, skin *AF* was related to cumulative metabolic stress and transient hyperglycemia in hemodialysis patients and historical glucose exposure in peritoneal dialysis patients.[34]

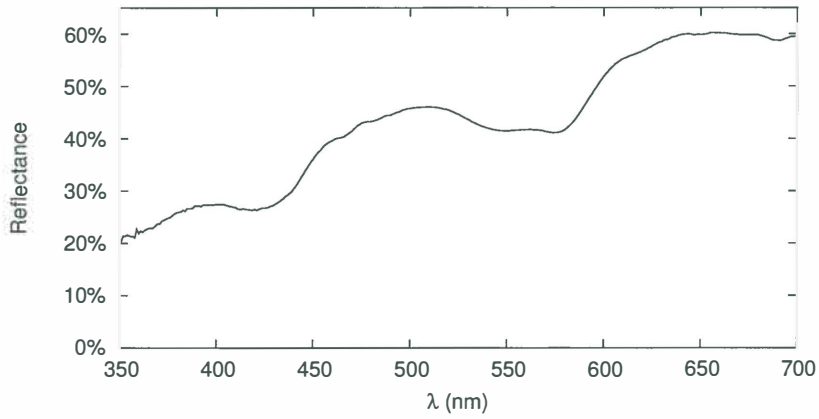

Figure 1.6: *A typical reflectance spectrum as measured from a healthy Caucasian subject.*

Research questions to be explored

Light propagation in tissue and absorption by skin chromophores

Upon illumination of the skin, a small part of the light, approximately 4 – 7%,[35] is specularly reflected due to the differences in refractive indices of tissue (~1.5) and the surrounding air (1.0). Most light is entering the skin. Due to multiple scattering, the direction of the light is changing and part of the light may finally leave the skin as diffusely reflected light. The remaining light will be absorbed by various molecules, of which some will subsequently emit fluorescent light with a higher wavelength.

Because of the wavelength dependent ability of molecules to absorb light, the amount of light that leaves the skin is also wavelength dependent. Therefore, a reflectance spectrum of human skin shows some very distinct characteristics. A typical reflectance spectrum is shown in Figure 1.6. The vertical scale represents the amount of light that was measured as a percentage of the amount of light that was measured from a non-absorbing reflectance standard. This percentage is plotted for each wavelength in the near UV-A and visual range of the spectrum. It can clearly be seen that the reflectance is lower for lower wavelengths. This is mainly due to melanin absorption.[35–37] The features in the 400 – 450 nm and 500 – 600 nm ranges are characteristic for hemoglobin absorption.[38]

Absorption by melanin and hemoglobin may be important confounding factors in

the measurement of skin fluorescence. Moreover, this effect may become more distinct when absorption fluctuates in a relatively short period of time. Potential causes for such fluctuations include sun tanning, intake of food and dialysis treatment. Part of this problem may have been solved by a technique that was suggested and validated by Coremans et al.[39] They found that the fluorescence signal could be corrected for intrinsic absorption by dividing the signal by the reflected excitation light. The principle was based on the assumption that whenever the measured fluorescence signal is decreased because of absorption and scattering, the amount of reflected excitation light will be decreased as well. By dividing these two quantities, the effect of changes in absorption and scattering is partly compensated. This technique was also applied in the AGE Reader, where skin AF is calculated as the emission light intensity, divided by the reflected excitation light intensity, as in Equation 1.2. Although Coremans et al. used this technique in heart tissue, where melanin absorption is not an issue, it works reasonably well in human skin, provided that the absorption is not too strong. However, it was shown that if reflectance of the UV excitation light is below 10%, absorption was increasingly influencing the value of skin AF.[40]

Specular reflectance

Normalizing the fluorescence signal using the measured reflectance in the excitation range also introduced a disadvantage. As stated above, the measured reflectance also includes a fraction of specular reflection, causing an artificial increase of the denominator in Equation 1.2, which is not present in the numerator that consists of only emission light. This effect will yield a lower value of skin AF, depending on the relative amount of specular reflection. With equal specular reflection for all skin colors, the relative contribution increases if the diffuse reflection is lower. Moreover, the refractive index of the stratum corneum, the first layer of the skin, may increase because of high melanin content, especially in subjects with a darker skin color.[41] A higher refractive index of this outer layer of the skin causes more specular reflection. Therefore, we hypothesize that more relative specular reflection is expected in subjects with darker skin color.

Measurement in subjects with dark skin colors

An important current limitation of the AGE Reader was that values as measured on subjects with darker skin colors were significantly lower than on subjects with a fair

skin color. Moreover, this effect was stronger for darker skin colors. This is probably largely due to the effect that melanin absorbs more of the reflected excitation light than of the emission light, although specular reflectance may play a role as well. It was found that, in general, reliable skin *AF* values can be obtained only if the skin reflectance in the UV excitation range exceeds 10% which is comparable with Fitzpatrick skin photo type I – IV. Because of this, it is hard to judge skin *AF* values in subjects with a dark skin color. However, in a pilot study, we have seen that even in these subjects, skin *AF* was increased in patients with DM-related complications. Therefore, it seemed possible to adapt skin *AF* values in such a way that skin *AF* can be used for detection or prediction of persons with complications in all subjects, independent of skin color.

Influence of different fluorophores

Another potential disturbing factor in assessing cardiovascular risk by using fluorescence properties of AGEs, is that also other compounds in the skin exhibit fluorescence. Some fluorophores that may directly contribute to the total fluorescence signal have already been listed in Table 1.1. It is not known yet how much these fluorophores contribute to the total fluorescence signal. Noordzij et al. recently performed ischemia (prolonged cold water exposure of the arm) and post occlusive reactive hyperemia experiments in human volunteers.[42] They found an increase of 10% and a decrease of 2.3% for these conditions respectively, apart from the influence of extra absorption by an increased blood volume fraction. This may be partly due to changes in levels of reduced nicotinamide adenine dinucleotide (NADH). Moreover, other fluorophores may inhibit the fluorescence signal by absorbing emission light originating from AGEs. These include porphyrins, elastin crosslinks, flavin adenine dinucleotide (FAD), flavins and phospholipids. However, in the end, a more important question is whether fluorescence by other fluorophores actually interferes with the assessment of cardiovascular risk.

Also interesting in this respect is the question whether the applied broad excitation spectrum (approximately 350 – 410 nm) is optimal for assessing cardiovascular risk. The use of a more specific (narrower) peak, or a combination of some peaks may improve this assessment if the fluorophores are excited in optimal proportions. The same question may hold for the wide range in which the emission is measured (420 – 600 nm). Together with the AFR, also an excitation-emission matrix scanner (EEMS) was developed at the Department of Biomedical Engineering to further investigate

whether a selective choice of wavelengths would be more related to patient characteristics. In the EEMS, a xenon lamp in combination with a tunable monochromator was used as a light source instead of a UV tube. With this setup, the skin can be illuminated with light of wavelength bands as narrow as 10 nm. The skin illumination and light detection in the EEMS were kept similar to those of the AFR.

Even if all tissue fluorophores are known and could be measured independently, skin *AF* measurements may still suffer from changes in plasma fluorophore composition. The reflection spectrum as shown in Figure 1.6 clearly shows the influence of hemoglobin absorption in the emission range, indicating that the AGE Reader may also measure fluorophores in the vascular system and the surrounding interstitial tissue. Therefore, in extreme cases, skin *AF* values may temporarily rise after the consumption of meals with heavy AGE load.[43]

Reference values

The assessment of increased cardiovascular risk may be aided by more knowledge on baseline *AF* values for healthy subjects. Skin *AF* is known to increase with age in years and a certain *AF* value may be high for a young person, whereas the same value may be relatively low for an older person. The by far most important risk factor for cardiovascular disease is subject age. In developed countries, cardiovascular events are estimated to be the cause of death in over 35% of mortalities.[44] However, in many cases we may also be interested in the relative risk of a patient as compared to subjects of the same age. The description of reference values as a function of age in healthy subjects is of interest in these situations.

Aim of the thesis

The aim of this thesis was to further improve the measurement of skin *AF* from a biomedical engineering perspective. This approach was used in the first place to improve the clinical applicability of the measurement of skin *AF* with the AGE Reader. The specific questions to be answered were whether the use of a narrower excitation peak of a specific wavelength could improve the AGE Reader, what reference values of skin *AF* can be expected in healthy subjects and how skin *AF* can be measured reliably in subjects with a dark skin, and finally to study the effect of changes in plasma fluorescence on skin *AF* and skin color as measured during hemodialysis.

Rationale

In Chapter 2, the role of the excitation wavelength as used for skin *AF* measurements was studied. Since AGEs have an essential role in the development of DM related chronic complications, we expected differences in the excitation or emission spectra as measured from the skin of subjects with DM-related complications as compared to DM patients without complications and control subjects. We addressed the issue whether the use of more specific wavelengths would improve the assessment of the risk for these chronic complications. In this chapter, excitation/emission matrices were used to explore these effects.

Chapter 3 shortly addresses the similarity of skin *AF* values as obtained with the AGE Reader and its earlier version, the AFR. Measurements on subjects with both instruments are compared.

In Chapter 4, reference values of skin *AF* for Caucasian healthy control subjects were investigated. Based on earlier results, we expected to find a linear increase of skin *AF* with subject age and also some increase for current smokers because of an increased AGE load. We extended these measurements to subjects with a lower age, because few measurements had been performed earlier on young subjects (age < 20 years): we expected the linear trend to continue for these ages.

Chapter 5 addressed skin *AF* measurements on subjects with a dark skin color. Various characteristics of reflectance spectra as measured with the AGE Reader were derived that correlated with the effect of skin color on *AF*. The characteristics were combined with expected values of skin *AF*, as obtained in Chapter 4 for Caucasian subjects, to arrive at an empirical method to make the AGE Reader more generally applicable in persons with a darker skin color.

In Chapter 6, the influence of hemodialysis treatment sessions on AGE Reader measurements was explored. Hemodialysis was expected to have an impact on skin *AF* and the diffuse skin reflectance. The influence of plasma fluorescence on skin *AF* was also investigated. Furthermore, we aimed at describing the decrease in plasma fluorescence as a function of changes in the reflectance measurements.

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CHAPTER 2

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

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Abstract

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. 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.

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.

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 fluorescence (CLF) in skin biopsies and are classically determined by fluorescence at 440 nmupon 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 (*AF*).[24–27] With this AFR, a prototype of the current AGE Reader (DiagnOptics, The Netherlands), the skin on the volar side of the forearm was illuminated with light in an excitation range of 350 – 420 nm (maximum intensity at 370 nm), and emission in the range 420 – 600 nm was measured with a spectrometer. With these measurements, a strong correlation was not only found with CLF from dermal skin biopsies, but also with skin AGEs (pentosidine, N^{ϵ}-(carboxymethyl)lysine, N^{ϵ}-(carboxyethyl)lysine) from the same skin biopsies from DM patients, renal failure patients and healthy persons. These biopsies were taken from the same site as where the autofluorescence was measured.[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 2.2 (continuous line). As shown above, this broad and fixed excitation range already provides important clinical information. However, it is not known which excitation wavelengths provide this information. For the investigation of the influence of excitation wavelength within the current excitation

range, Excitation/Emission Matrices (EEMs)[35–37] can be used. 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 investigate the contributions of skin autofluorescence at excitation wavelengths within this range in various clinical conditions. This study will concentrate on EEMS measurements obtained from DM patients with and without DM-related chronic complications. Furthermore, the study will be limited to the excitation range of approximately 355 – 405 nm, as used in the AGE Reader, since this range already provides a very significant clinical parameter.

The aim of this study is first to determine whether specific excitation wavelengths within the currently used excitation range have to be preferred over the broad excitation peak for differentiation between diabetic and non-diabetic subjects or between diabetic subjects with and without DM-related chronic complications. And secondly, to evaluate whether differences in fluorescence emission occur that are associated with the presence of DM-related chronic complications.

Materials and methods

Subjects

EEMs 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 2.1. 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] Patients were classified as having no complications only if they were in good health, except for their DM status. Table 2.1 shows the characteristics of the five subject groups.

Table 2.1: Characteristics of the five groups. Where applicable, values are mean ± 1 s.d.

	DM type 1		DM type 2		Control
Complications	no	yes	no	yes	
N	19	17	21	16	24
Age (yr)	42.3 ± 4.5	43.2 ± 4.6	45.8 ± 4.7	47.5 ± 5.1	46.8 ± 3.3
Gender (M:F)	8:11	6:11	15:6	10:6	7:17
Diabetes duration (yr)	20 ± 11	28 ± 7	5 ± 1	9 ± 6	

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 (of approximately 4 cm^2) under an angle of 45°, as shown in Figure 2.1. This glass fiber is connected to a spectrometer (USB2000, Avantes, Eerbeek, The Netherlands) with a spectral range of at least 300 – 750 nm and a resolution of approximately 0.3 nm. The spectral sensitivity equals that of the AFR and current AGE Reader. All spectra are immediately converted to a spectral resolution of 1 nm. 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. Each measurement provides the full spectrum, including the excitation and emission ranges. Dark spectra were also obtained and subtracted for each measurement to correct for dark current deviations in the applied CCD detector. The shape of the peak at each selected excitation wavelength has a standard deviation of 6.5 and the Full Width at Half Maximum (FWHM) is approximately 10 nm.

erratum

In het proefschrift *Improving the measurement of skin autofluorescence, or working on the clinical applicability of skin autofluorescence from a biomedical engineering perspective* is per ongeluk pagina 21 onbedrukt gebleven. Op de weerzijde van dit blad staat alsnog de bedoelde tekst.

In the dissertation *Improving the measurement of skin autofluorescence, or working on the clinical applicability of skin autofluorescence from a biomedical engineering perspective*, page 21 has been left blank unintentionally. Please find the text for this page on the other side of this sheet.

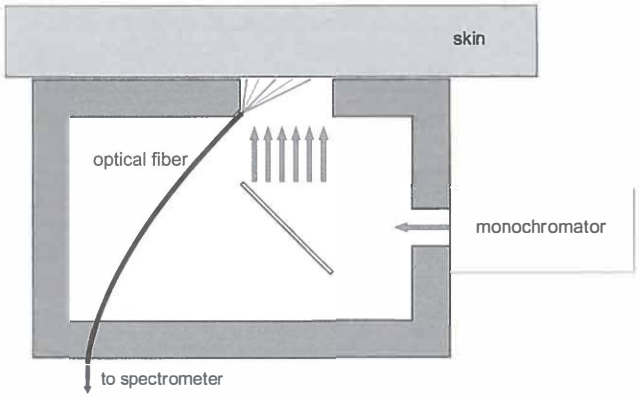

Figure 2.1: *Instrument schematic of the EEMS. UV excitation light from the monochromator is directed to the skin via a mirror. The emission and reflection light from the skin is guided to a spectrometer via an optical fiber, pointing at the measuring site at an angle of 45°.*

Analyses and statistics

Autofluorescence as obtained from the EEMS (AF_E) was defined for each measurement at a selected excitation wavelength separately and for all individual subjects as

$$AF_E(\lambda_{exc}, s) = 100\frac{\langle I_{em}\rangle(\lambda_{exc}, s)}{\langle I_{exc}\rangle(\lambda_{exc}, s)}, \tag{2.1}$$

where $\langle I_{em}\rangle(\lambda_{exc}, s)$ is the mean measured light intensity in the range 420 – 600 nm and $\langle I_{exc}\rangle(\lambda_{exc}, s)$ is the mean intensity in the range 300 – 420 nm, both determined from the spectrum that was obtained with the selected excitation wavelength λ_{exc} and for subject s. By defining AF_E as a ratio of emission and reflected excitation light, AF_E is compensated for variations in lamp intensity and partly for changes in skin color. The same method is used in the AGE Reader to calculate AF from a spectrum.[25, 26]

After calculating AF_E for all subjects and all excitation wavelengths, the mean, $\langle AF_E\rangle$, for subjects in the control group per excitation wavelength was used as a reference for all four DM subject groups.

For comparison between AF_E values of DM subject groups and the control group, we introduced relative autofluorescence for each excitation wavelength for the DM

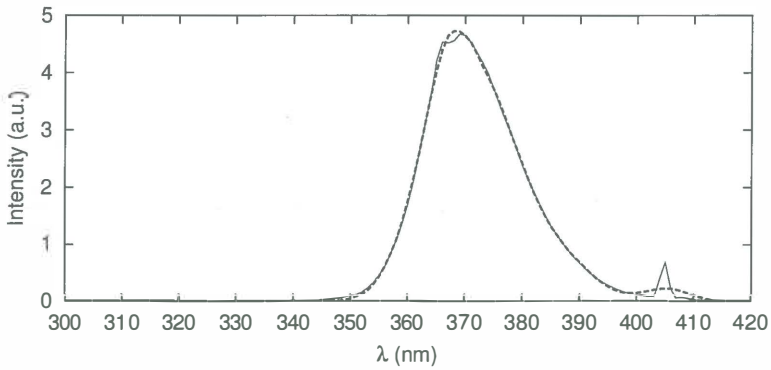

Figure 2.2: *Spectrum of the UV-A light source as used in the AFR and the AGE Reader (continuous line) and a fit using a weighted summation of EEMS responses of separate excitation peaks (dotted line). The small peaks at 366 nm and at 405 nm are caused by mercury emission within the lamp.*

patients as

$$AF_{E,rel}(\lambda_{exc}, s) = \frac{AF_E(\lambda_{exc}, s)}{\langle AF_E \rangle (\lambda_{exc}, control)} \tag{2.2}$$

where $AF_E(\lambda_{exc}, s)$ is the AF_E of a patient in the DM subject group for excitation wavelength λ_{exc} and $\langle AF_E \rangle (\lambda_{exc}, control)$ is the mean AF_E of all subjects in the control group for that excitation wavelength. 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 2.2. In order to derive an analysis for this light source as well, a weighted summation of measured responses on excitation at given wavelengths was used. The AF that would have resulted from the blacklight, AF_{BL}, per subject was calculated using

$$AF_{BL} = \frac{\sum w_i AF_i}{\sum w_i}, \tag{2.3}$$

where AF_i is AF_E as calculated from excitation peak i (where each i represents one of the excitation maxima between 355 and 405 nm) 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

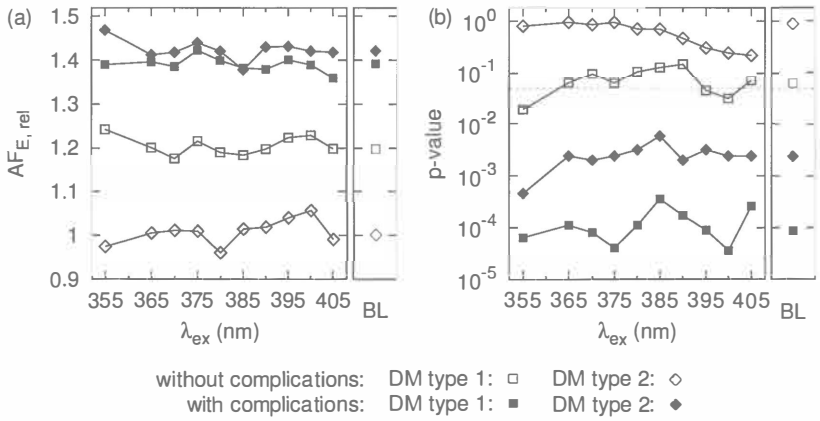

Figure 2.3: *(**a**) Mean relative autofluorescence values for the separate excitation peaks ($AF_{E,rel}(\lambda_{exc})$) and the broad excitation peak from the blacklight (BL) as used in the AGE Reader. (**b**) Statistical significance (Mann–Whitney U-test) of the differences between AF_E values of subjects in the respective subject groups and the control group. The dotted line in (**b**) represents a value of $p = 0.05$.*

Figure 2.2 (dotted line) for comparison. The Mann–Whitney U-test calculations were also performed for AF_{BL}.

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, $AF_E(\lambda_{exc}, s)$, were obtained using Equation 2.1 for all subjects and for excitation wavelengths in the range 355 – 405 nm in steps of 5 nm. Figure 2.3(a) shows the mean relative $AF_E(\lambda_{exc})$ values for the four groups, as obtained by Equation 2.2. The standard deviation of the AF values within each group ranged

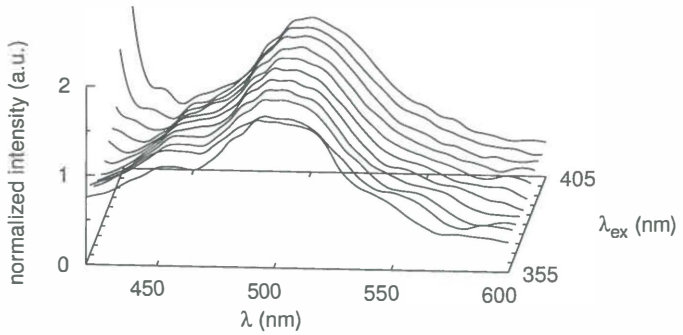

Figure 2.4: *Emission peaks from the average spectra of the control group for increasing excitation wavelength. The spectra are normalized for emission.*

between 18% and 36% of the mean value. Instead of error bars, Figure 2.3(b) 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 2.3 was used to calculate results for the broad excitation peak of the AGE Reader. Figure 2.3 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 2.3 is not significant ($p > 0.05$) and may be caused by the longer diabetes duration of the DM type 1 patients.

Normalized spectra

The emission peaks of the normalized spectra for the control group are displayed in Figure 2.4. The shape of the spectrum is slightly changing for different excitation wavelengths. However, the maximum intensity remains at approximately 500 nm,

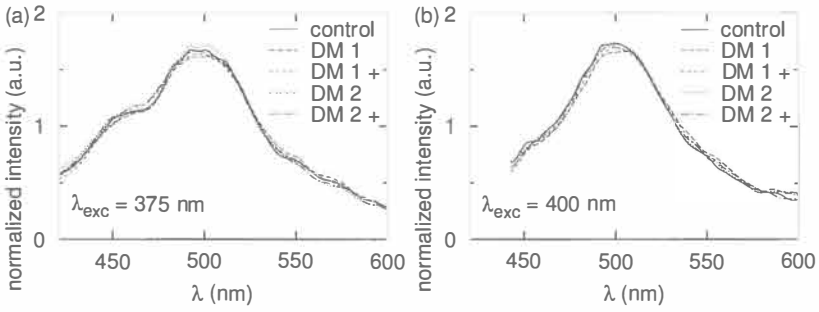

Figure 2.5: *Emission peaks from the average spectra of the five groups for λ_{exc} = 375 nm (**a**) and λ_{exc} = 400 nm (**b**). In (**b**), the spectrum starts at 443 nm as below that wavelength, the excitation peak is still present.*

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 2.5 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.

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 relative AF values and significances thereof for all excitation wavelengths. As the excitation spectrum of the AGE Reader is sufficiently modelled by a combination of the investigated excitation spectra (Figure 2.2), we may conclude that about the same relative AF values 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 within the current range, 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, the fluorophores that contribute to emission in this range seem to be equally increased in the groups of patients with complications.

Figure 2.3 shows some small variations in relative AF for both type 1 DM patient groups at 375 nm, which might be interpreted as an indication of influence of the excitation wavelength on $AF_{E,rel}$ for these groups. However, these variations in $AF_{E,rel}$ are much smaller than the influence of complications on $AF_{E,rel}$. Furthermore, it can be noted from the figure that the relative AF values are highest for the group of DM type 2 patients with complications, although these are not the most significant. This is mainly the result of somewhat more variation within this group.

The levels of glycated hemoglobin, HbA_{1c}, have not been used in the present study. Earlier results have shown that AF is a much stronger marker than HbA_{1c} in assessing chronic complications,[24, 27, 33, 34] probably due to the high turnover of HbA_{1c} in blood. For reference, the levels of HbA_{1c} were 5.1 ± 0.3 % in the control group (normal range 4.2 – 6.0 %) and 7.8 ± 1.5 % in the various DM groups and had a variation between the DM groups of less than one standard deviation of the mean.

It is well known that many fluorophores exist in the skin.[22, 38] From the field of AGEs, it has become clear that many AGE molecules may also contribute to autofluorescence.[39] Therefore, the observed broad emission spectra as seen in the normalized spectra could be expected, even within the limited range we investigated. It should be noted that, although various fluorophores may have very different excitation maxima, variation of the excitation wavelength only yields a few changes in the shape of the emission spectrum. The emission spectra upon excitation with 375 nm (which are shown in Figure 2.4(a) show a peak at approximately 460 nm, which is not as prominent in the emission spectrum upon excitation with 405 nm (Figure 2.4(b)). However, this same shape is visible in the emission spectra from all 5 groups. This is probably due to the fact 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.[40]

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.[2, 22, 41] We speculate that the same pool of fluorophores causes fluorescence in the current range in healthy subjects as in DM patients, since only the amount of fluorescence 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, 42, 43] 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 the presence of DM-related chronic complications, as has been reported before.[27, 34] The study of Lutgers et al.[27] reported an increased AF for type 2 DM patients without chronic complications, while our study showed no increased AF in this group. 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 an almost constant increase of AF values 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 DM.

Conclusions

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. The shape of the emission spectra did thereby not show any influence of DM or DM-related complications. These results therefore show the validity of a broad excitation wavelength range, such as applied in the AGE Reader.

Acknowledgments

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Competing interest

R. Graaff and A.J. Smit are also founders of DiagnOptics B.V., manufacturer of the AGE Reader (www.diagnoptics.com).

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CHAPTER 3

Comparison of the AutoFluorescence Reader and the AGE Reader

Abstract

The AGE Reader is a fully automated version of the AutoFluorescence Reader (AFR). During its development, special care has been taken to obtain instruments that yield the same values of skin autofluorescence (*AF*). This study shortly reviews the similarities and differences between both instruments and provides a direct comparison of skin *AF* values as obtained with both instruments. It is concluded that both instruments yield similar skin *AF* values over a broad range of values. The AGE Reader can directly be linked to clinical validation studies that were performed with the AFR.

Introduction

The AGE Reader, as developed by DiagnOptics Technologies B.V., Groningen, The Netherlands, has been based on the AutoFluorescence Reader (AFR) as developed by the Departments of Biomedical Engineering and Vascular Medicine of the University Medical Center Groningen as described in Chapter 1. The AGE Reader is a fully automated version of the AFR, where all components have been integrated and communicate with the driving and analyzing software on an external personal computer. Special care has been taken to obtain instruments that yield the same values of skin autofluorescence (AF) on the same subject. The optical design, choice of light source, the spectrometer setup and analysis steps have all been kept similar. In this study, both instruments are shortly described and the similarities and differences between both instruments are discussed. Then, a direct comparison of skin AF values as obtained with both instruments is provided. The aim was to study whether both instruments yield similar skin AF values over a broad range of values and, with that, whether the AGE Reader can directly be linked to clinical validation studies that were performed with the AFR.

Setup of the devices

In both instruments (AFR and AGE Reader) the excitation light source is a UV-A blacklight tube, with a peak wavelength of 370 nm. The skin of the subject is illuminated perpendicularly on an area of approximately 4 cm^2. A non-contact optical fiber detects the emission and reflected excitation light at an angle of 45°. The detection fiber is pointed at the center of the illuminated surface. The illuminated surface area exceeds the detection area, such that the size of the illuminated area does not influence the results anymore. A spectrometer and computer software are used to analyze the spectra. A measurement is performed without illumination to compensate the measurement for dark current in the detector. A measurement on an internal white reflection standard is performed as a reference to calculate a reflectance spectrum. To calculate the skin AF value, the average measured intensity in the 420 – 600 nm emission range is divided by the average measured intensity in the 300 – 420 nm excitation range and multiplied by 100.

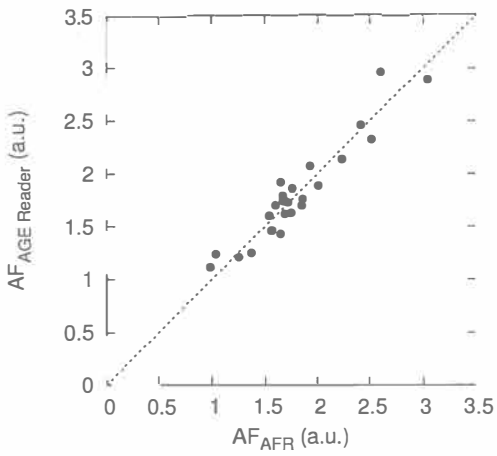

Figure 3.1: Results of skin AF values as measured with the AFR (horizontal axis) and AGE Reader (vertical axis). The dotted line represents equal values for both instruments ($y = x$).

Relevant differences between the devices

In the AGE Reader, a Borofloat-glass window (Schott, Jena, Germany) was introduced in the sample opening for protection of the interior. This glass window has been selected for exhibiting only very low fluorescence. Furthermore, a rubber mat is applied for better shielding of environmental light and subject comfort and to aid proper cleaning. Finally, instead of a rectangular opening as in the AFR, the AGE Reader has a square measurement window.

Methods

Subjects

For a direct intra-individual comparison between the AFR and the AGE Reader, skin *AF* was measured in 25 healthy and diabetic volunteers, representing a broad range of skin *AF* values. Subject age ranged from 20 to 84 years. With both instruments, skin *AF* was measured three times on adjacent sites of the volar side of the same forearm. Mean values of skin *AF* of each subject were used for the analysis. These

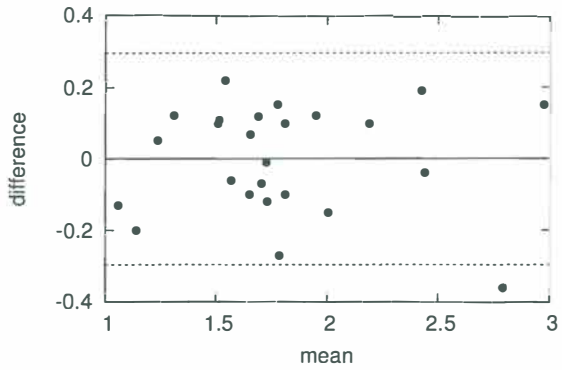

Figure 3.2: *Bland–Altman plot, showing the similarity between both instruments.*

measurements were also used in Chapter 4 on reference values of skin *AF* in healthy Caucasian subjects.[1]

Statistical analysis

Skin *AF* values with both instruments were compared using a Bland–Altman plot. The mean relative error was calculated by dividing the standard deviation of the differences by the mean value of *AF*, multiplied by the square root of 2.

Results

Mean values of skin *AF* from 3 measurements per subject were calculated for the AFR and the AGE Reader. For the total group, mean *AF* ± 1 standard deviation was 1.80 ± 0.47 for the AFR and 1.80 ± 0.48 for the AGE Reader. The individual values are presented in Figure 3.1, where the horizontal axis represents skin *AF* as measured with the AFR and the vertical axis represents values from the AGE Reader.

A Bland–Altman plot was generated to show the similarity of both instruments, see Figure 3.2. The mean difference (*AF* value obtained with the AFR minus the *AF* value obtained with the AGE Reader) is 0.0004. The standard deviation of the difference is 0.15. The mean relative error is 0.059.

Conclusion

Although slight differences exist in some components and dimensions between the AFR and the AGE Reader, both instruments yield similar results. Means and standard deviations of skin *AF* values from both instruments are equal. The Bland–Altman plot showed good concordance between results of the AFR and the AGE Reader, with a mean relative error of 6%. Validation of skin *AF* values as measured with the AFR may be used for validation of the AGE Reader as well.

Acknowledgements

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CHAPTER 4

Reference values of skin autofluorescence

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*Diabetes Technol Ther 2010;**12**:399–403*

Abstract

Background — Skin autofluorescence (AF) as measured with the AGE Reader is a non-invasive prognostic marker in diabetes mellitus and other diseases with increased cardiovascular risk. This study provides reference values of healthy Caucasian control subjects as a function of age, tobacco smoking and gender.

Methods — The results of skin AF measured in 428 healthy Caucasian control subjects by the AGE Reader ($n = 211$) and its non-automated, but otherwise similar predecessor, the AutoFluorescence Reader ($n = 217$) were analyzed. Linear regression analysis was performed to obtain reference values for skin AF as a function of age. Further analysis was performed on the effect of tobacco smoking ($n = 96$) and gender.

Results — Skin AF was described by a linear increase with age of approximately 0.023 arbitrary units (AU) per year for subject age up to 70 years. Tobacco smoking was associated with an absolute increase of skin AF by 0.16 AU ($p < 0.01$), without a significant further increase with age ($p = 0.17$). Gender had no influence on skin AF in non-smokers. In current smokers, female subjects had a 0.2 AU higher skin AF than male subjects ($p = 0.02$), with no further age related increase.

Conclusions — The present results provide reference values of skin AF for healthy Caucasian control subjects over a broad age range. A major contribution of age and some interaction of smoking and gender were observed, resulting in reference values of skin AF suitable for clinical settings and future studies.

Introduction

The amount of accumulated collagen-linked advanced glycation endproducts (AGEs), assessed in skin samples, has previously been shown to be an independent predictor for development and progression of diabetes complications.[1,2] More recently, noninvasive skin autofluorescence (*AF*) appeared also to be a predictive marker for complications in diabetes mellitus and other diseases with increased cardiovascular risk.[3–14] In one of these publications, skin *AF* values for healthy controls were roughly linearly related to age and were reported for the decades between 40 and 79 years. Levels were higher in smokers.[7] So far, no other reference values, also for other age groups or possible confounders like gender or smoking, have been reported. In these studies, skin *AF* was measured with the AutoFluorescence Reader (AFR). DiagnOptics Technologies (Groningen, The Netherlands) further developed this AFR into a fully automated but otherwise similar instrument and called it the AGE Reader.[6] The AGE Reader further simplifies the measurement of skin *AF*. With an increasing number of ongoing clinical studies that use the AGE Reader, there is a growing demand for standard reference values of skin *AF*.

The aim of the present study was to provide reference values of skin *AF* for control subjects over a broader age range. Furthermore, the effects of age, tobacco use and gender have been investigated.

Subjects and Methods

Instrumentation

The skin *AF* results were obtained with the AFR and the AGE Reader. The AGE Reader is a fully automated version of the AFR with a built-in spectrometer. In both instruments (AFR and AGE Reader) the excitation light source is a similar ultraviolet-A blacklight tube, with a peak wavelength of 370 nm, which illuminates ~4 cm^2 of the skin on the volar side of the forearm. A non-contact optical fiber detects the emission and reflected excitation light at an angle of 45°. Using a spectrometer and computer software, the intensity spectrum, of which a typical example is shown in Figure 4.1, is analyzed. Skin *AF* is calculated as the ratio between the total emission intensity (420 – 600 nm) and the total excitation intensity (300 – 420 nm), multiplied by 100, and is expressed in arbitrary units (AU).

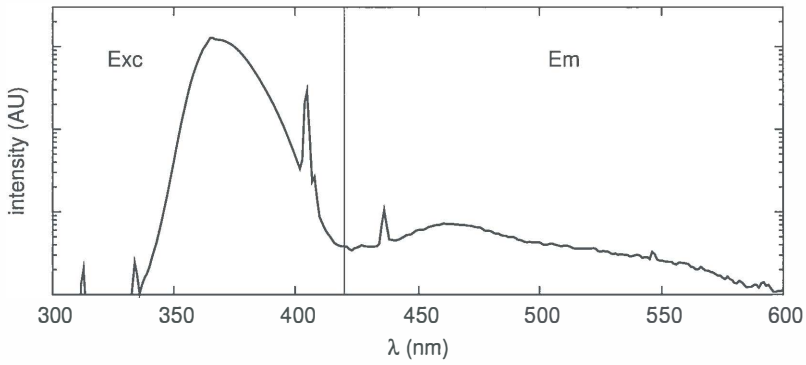

Figure 4.1: *Typical example of an intensity spectrum as detected from the skin. "Exc" is the excitation part of the spectrum (300 – 420 nm); "Em" is the emission part (420 – 600 nm).*

Although also the optical path and parts of the AGE Reader have been designed to be similar to that of the AFR, some differences between the two instruments exist. The AGE Reader has a Borofloat-glass window (Schott, Jena, Germany) in the sample opening for protection of the interior. This glass window has been selected for exhibiting only very low fluorescence. Furthermore, a rubber mat is applied for better shielding of environmental light, subject comfort and to aid proper cleaning. To arrive at an instrument that provides results in agreement with the AFR, a direct intraindividual comparison between the AFR and an AGE Reader was performed by DiagnOptics Technologies, using a limited number of healthy individuals and subjects with diabetes ($n = 25$) with a broad range of age and skin *AF* values. Figure 4.2 shows the results from this substudy.

Measurements in the first group

In the previous study, skin *AF* values were collected using the AFR from a group of 231 Caucasian subjects.[7] The study is limited to Caucasian subjects because dark skin may influence skin *AF* assessment.[6] Skin *AF* was measured at three positions on the volar side of the lower arm, and the mean of these three measurements was used in the analysis. Besides skin *AF* values, information was obtained on age, gender, body mass index, current smoking status, and systolic and diastolic blood pressure. Subjects were regarded as non-smokers if they did not use tobacco within the last

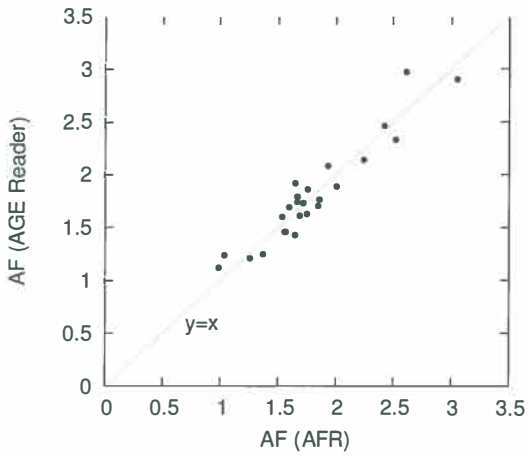

Figure 4.2: Skin AF as measured with the AGE Reader compared to the AFR. R^2 of a fit-line (not drawn) is 0.90.

year. Only subjects with no known history of diabetes, cardiovascular events, or renal dysfunction (creatinine values within reference values of 70 – 110 μmol/L) were included. As the subjects were recruited from the outpatient clinic for presurgery screening, they were further classified according to the American Society of Anesthesiologists (ASA) score (1 – 5). In the present study, only the control subjects, who had an ASA score of 1 or 2 (ASA 1 = normal healthy subjects; ASA 2 = subjects with a mild systemic disease), were included. The 20 persons with ASA score 3 were not included in the current analyses.

Measurements in the additional group

In addition to the measurements described above, we used the AGE Reader to measure skin *AF* in a group of 281 Caucasian volunteers with a broad age range. Using a self-administered questionnaire, information was obtained on age, gender, and current smoking status. Further items assessed in the questionnaire were history of hypertension, diabetes, stable coronary artery disease, acute coronary syndrome or coronary revascularization or intervention, stroke, and renal dysfunction. Subjects were excluded from this study if one of these items were positive or in case of pregnancy or use of body lotions or creams on the measuring site.

Analyses

The AGE Reader and AFR are similar instruments and should therefore generate the same results. Data of both groups were compared to evaluate this similarity. Because tobacco smoking influences skin AF,[7, 14] only nonsmokers were included in this first comparison. After correction for subject age, both groups were compared using Student's t-test. After the similarity of both instruments was established, results from both groups were combined for the further assessment of skin AF. Least squares linear regression analysis was performed on the total group, to obtain reference values for skin AF in a healthy population as a function of age. Subsequently, the effect of tobacco smoking was analyzed, after correcting for subject age. Finally, the effect of gender was investigated for the current smokers and the nonsmokers, by assessing the gender-dependent residual after correction for age using the respective age fits for smokers and nonsmokers.

Results

Skin AF values of the non-smokers as obtained with the AFR and the AGE Reader are shown in Figure 4.3. Different symbols were used to show which of the instruments was used for each measurement. For the linear regression fits, only subjects less than 70 years old were included. In both groups, skin AF increases equally with subject age. The offset of 0.03 AU between the two regression lines is not significant ($p = 0.26$).

Group characteristics

For the measurements in the previously reported reference group,[7] 211 subjects (129 female and 82 male) were included. Age was 51 ± 17 years (mean ± s.d.) and ranged from 17 to 91 years. In this group, 32% of the subjects were current smokers (40 female and 28 male). For the measurements in the new cohort, 217 subjects (119 female and 98 male) were included. Age was 27 ± 17 years (mean ± s.d.) and ranged from 1.5 to 69 years. In this group, 13% of the subjects were current smokers (16 female and 12 male).

From here on, both groups have been combined into one large group of 428 subjects (248 female and 180 male). Age was 38 ± 21 years (mean ± s.d.) and ranged

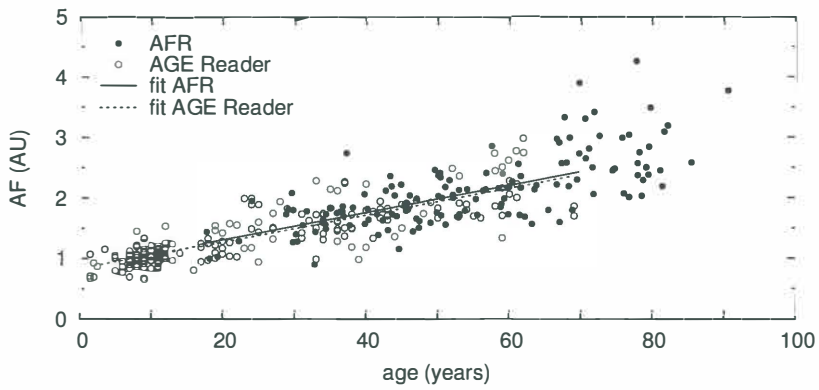

Figure 4.3: *Skin AF as a function of subject age. The data and linear regression fits are obtained from the AFR group (solid symbols and solid line) and the AGE Reader group (open symbols and dashed line). The fits are determined using least squares fitting.*

from 1.5 to 91 years. In the total group, 22% of the subjects were current smokers (56 female and 40 male).

Influence of age and smoking status

Skin AF values for the non-smokers and the current smokers of the total group are shown in Figure 4.4. Skin AF for the non-smoking subjects can be described as $0.023 \times A + 0.83$ ($R^2 = 68\%$), where A is subject age in years. The s.d. around this fit is 18% of the fit-value, independent of age. The current smokers have an increased skin AF compared to the non-smokers, which can be described as $0.029 \times A + 0.78$ ($R^2 = 41\%$). The s.d. around this fit is 23% of the fit-value, independent of age. The age related increase in skin AF was not significantly different between current smokers and non-smokers ($p = 0.12$). The effect of smoking on the skin AF value is given by an absolute increase, which is 0.16 AU ($p < 0.01$). The difference between smokers and non-smokers within each age group is only significant for the age groups 30 – 40 and 60 – 70 years.

When both groups (non-smokers and current smokers) are combined, skin AF of the total group can be described as $0.024 \times A + 0.83$ ($R^2 = 60\%$). This fit is not significantly different from the fit for the current non-smokers only ($p = 0.17$). The s.d. around the fit is 20% of the fit-value, independent of age.

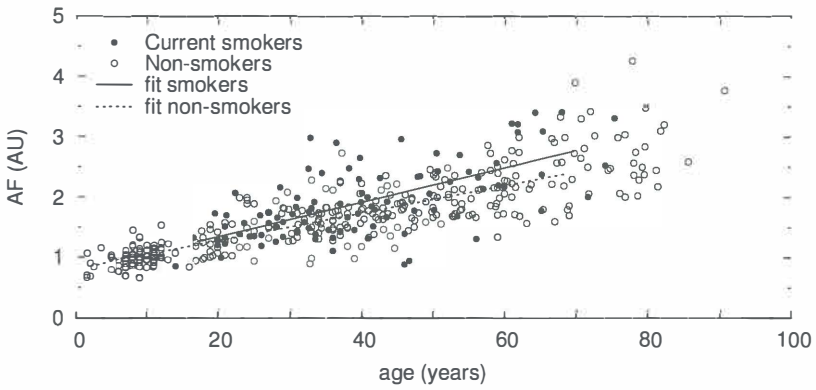

Figure 4.4: *Skin AF for the current smokers (solid symbols and solid line) and the non-smokers (open symbols and dashed line) of the total group as a function of subject age. The fits are determined using least squares fitting.*

The mean skin AF values per age group of 10 years are summarized in Table 4.1 for reference. The increase in skin AF per 10 years is significant for most age groups as indicated in Table 4.1.

Influence of gender

Within the non-smokers group, no effect of gender on skin AF was found. Within the current tobacco smokers, the female subjects had a higher skin AF than the male subjects. The effect of gender on the current smokers as studied up to 70 years of age was not dependent on age and was quantified as an absolute increase of 0.2 AU ($p = 0.02$). Female smokers had skin AF values of 0.24 higher than non-smokers ($p < 0.01$); male smokers had skin AF values of 0.04 higher than non-smokers ($p = 0.56$).

Discussion

This study provides reference values for skin AF with the AFR and the AGE Reader, which provided values for skin AF that are in agreement with each other. Skin AF can be described as a linear function of age over a broad age range up to the age of 70 years, with a remarkably constant degree of variation.

Table 4.1: *Mean skin AF values and group size per age group for the entire dataset. Values are mean values ± 1 s.d., in arbitrary units. Significance between subsequent age-groups is indicated as p-values from a Student's t-test.*

Age group (years)	*AF*	Number	*p*
0 – 10	0.97 ± 0.17	45	< 0.01
10 – 20	1.11 ± 0.20	50	< 0.01
20 – 30	1.53 ± 0.30	62	< 0.01
30 – 40	1.73 ± 0.42	86	0.21
40 – 50	1.81 ± 0.36	72	< 0.01
50 – 60	2.09 ± 0.36	64	< 0.01
60 – 70	2.46 ± 0.57	45	0.12
70 – 80	2.73 ± 0.55	27	0.51
80+	2.71 ± 0.44	5	

In the literature, a linear relation between skin *AF* and subject age has often been suggested.[15–17] With the results of the present study, we also constructed exponential and other regression fits. However, these yielded no better results than linear fits. For practical and clinical use, the given linear fits sufficiently describe the relation of skin *AF* with subject age.

Tobacco smoking increases AGE accumulation and skin *AF*.[7, 14, 18] In the present study population, skin *AF* was increased in current smokers compared to non-smoking subjects by an absolute increase of 0.16 AU. We expected that the effect of smoking is increasing with age, because older smokers presumably have been smoking longer and have a higher number of pack-years than the younger subjects and may thus have had more and longer exposure to exogenous AGEs in tobacco smoke. However, in our subject group, adding the current smokers (22% of all subjects are current smokers) to the total group results in no significant increase of average age-corrected skin *AF*.

In the present study, the number of smokers was 22%, which may be different in other studies. A formula may be constructed based on a weighted summation of the fits that were found in this study for non-smokers and current smokers. Reference values of skin *AF* can thus be adapted to the numbers of current male and female tobacco smokers who are present in the population of interest.

It should be noted that both age and smoking status should be taken into account also in populations of patients suffering from diabetes or diabetes-related complications because smoking status has been shown to be independently and positively

related to skin *AF*, in healthy subjects as well as in patients with diabetes.[7]

In our previous study, no difference was found in skin *AF* values for gender in control subjects.[7] In the current study, we neither found differences in skin *AF* between the female and male non-smokers. However, within the group of current smokers, female subjects had a higher skin *AF* than male subjects after correction for age. This suggests that women may suffer more from the effect of smoking than men, an effect that is also seen in chronic obstructive pulmonary disease.[19]

Food may have a small influence on skin *AF*: one study reported a 8.7% increase of skin *AF* in healthy subjects 2 h after a high fat breakfast (600 kcal) with also a considerable AGE content.[20] The effect of this meal barely decreased after 4 h. In a study where food intake was not restricted, the variation of skin *AF* over the day was 5%.[6] If food intake would have had an influence in the present study, this influence would have been randomly distributed over the subjects. The reference values of the present study can therefore be used for measurements in non-fasting subjects.

Within each age group of 10 years, the s.d. of the mean is approximately 20%. Because the coefficient of variation in skin *AF* for a single measurement is approximately 5%,[6] the remaining variation between subjects can be explained as variation in the amount of accumulated skin AGEs and therefore their relative risk for development of cardiovascular disease. Tissue AGE accumulation has been proposed as a carrier of so-called metabolic memory and the legacy effect.[21] The variation in skin *AF* may at the same time represent the interindividual differences in metabolic memory and vascular damage.

Only subjects with an age below 70 years were included in the linear fit analysis. Although the linear regression for skin *AF* seems to continue beyond this age, we noted that some elderly subjects have a more increased skin *AF* value. Higher skin *AF* values may be expected in elderly subjects because they may develop complications related to age. On the other hand, it may be expected that the elderly may have a relatively lower skin *AF* value because lower skin *AF* is associated with longer survival. These two effects explain the wider distribution and uncertainty, which is the reason why we excluded this age group from the linear regression analysis.

The current study is limited to Caucasian subjects. Therefore, our results may not be applicable to subjects with more pigmented skin. Improvements of the AGE Reader are in progress to enable reliable skin *AF* measurements in those subjects.

In conclusion, the described relation of skin *AF* with age, smoking status, and gender as shown for Caucasians provides reference values to apply in clinical settings and future studies.

Author Disclosure Statement

R.G. and A.J.S. are also founders and stockholders of the university spin-off DiagnOptics Technologies B.V., manufacturer of the AGE Reader (www.diagnoptics.com). M.K. is a Ph.D. student at the Department of Biomedical Engineering whose study is partly financed by DiagnOptics Technologies B.V. H.L.L., C. de J., and T.P.L. declare no competing financial interests.

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CHAPTER 5

Skin color independent assessment of aging using skin autofluorescence

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Abstract

Skin autofluorescence (AF) for the non-invasive assessment of the amount of accumulated tissue Advanced Glycation Endproducts (AGEs) increases with aging. In subjects with darker skin colors, measurements typically result in lower AF values than in subjects with fair skin colors, e.g. due to selective absorption by skin compounds. Our aim was to provide a new method for calculating skin AF, yielding values that are independent of skin color. The deviation of skin AF of healthy subjects with various darker skin types ($n = 99$) compared to reference values from Caucasians showed to be a function of various parameters that were derived from reflectance and emission spectra in the UV and visible range (adjusted $R^2 = 80\%$). Validation of the new algorithm, based on these findings, in a separate dataset ($n = 141$) showed that results of skin AF can now be obtained to assess skin AGEs independently of skin color.

Introduction

Measuring skin autofluorescence (*AF*) is a non-invasive method for determining the amount of accumulated tissue Advanced Glycation Endproducts (AGEs). A significant correlation exists between skin *AF* and levels of skin AGEs like pentosidine, N^{ϵ}-(carboxymethyl)lysine (CML) and N^{ϵ}-(carboxyethyl)lysine (CEL), as obtained from skin biopsies: in a combined analysis of skin biopsy validation studies,[1] 76% of the variation in skin *AF* can be explained by variations in skin biopsy pentosidine levels.[2–4] Skin *AF* has shown to increase with age and is also an independent predictor of development and progression of complications in diabetes mellitus, renal failure and other diseases with increased cardiovascular risk.[3, 5–9] Skin *AF* is measured with the AGE Reader, from the mean emission in the 420 – 600 nm range upon UV-A excitation with a peak wavelength of 370 nm.

Skin *AF* measurements in subjects with darker skin colors (UV-reflectance below 10%) typically result in lower values than in subjects with fair skin colors.[10] It is not expected that these subjects have a substantially lower amount of AGEs. The lower *AF* values are therefore expected to be caused by different absorption of excitation or emission light by skin compounds and scattering effects, especially in the epidermis, and specular reflectance. The observed skin color dependence hinders reliable assessment of skin AGEs in subjects with darker skin color and inhibits the recognition of increased skin *AF* values.

Although literature provides some methods to describe the influence of absorbers and scatterers on skin color,[11–14] for the current study, we have chosen for an empirical approach by using parameters that are calculated from spectra that are measured individually in the UV-A and visible range (350 – 675 nm). The focus was to develop a model to adapt skin *AF* in healthy subjects for the influence of skin color. For this model, the main spectral characteristics of the strongest contributing absorbers, melanin, hemoglobin and bilirubin, have been used as a basis for finding significant parameters that may describe the lower skin *AF* values in subjects with a dark skin color.

In the current study, various parameters from the spectra are described, that may correlate with the decrease of *AF* for darker skin colors. With these parameters, multiple linear regression analysis was performed to find a model to describe the deviation of *AF* from an expected value. Based on this model, an algorithm to calculate skin *AF* has been constructed and subsequently validated using measurements on healthy subjects of various skin color.

Materials and methods

Measurement setup

Skin *AF* was measured with the AGE Reader (DiagnOptics Technologies B.V., Groningen, The Netherlands). A UV-A blacklight tube (F4T5BLB, Philips, Eindhoven, The Netherlands), with a peak wavelength of 370 nm is used to illuminate ~4 cm^2 of the skin on the volar side of the forearm. A spectrum of the light source is shown in Figure 5.1. A non-contact optical fiber (200 μm diameter) detects the emission and reflected excitation light from ~0.4 cm^2 at an angle of 45°. Using a spectrometer (AvaSpec_2048, Avantes, Eerbeek, The Netherlands) and computer software, the intensity spectrum is analyzed. The value of skin *AF* is calculated as the ratio between the total emission intensity (420 – 600 nm) and the total excitation intensity (300 – 420 nm), multiplied by 100 and is expressed in arbitrary units. Besides the skin *AF* measurement, UV-reflectance is calculated as the sum of the intensities of the reflected light from the skin in the range 300 – 420 nm, divided by the sum of intensities in the same range from a white reference standard, which is embedded in the AGE Reader and has been calibrated in situ against an external reflectance standard. Moreover, a complete diffuse reflectance spectrum is obtained, using a white LED as illumination source in the visible range. This LED is located directly under the detecting fiber. The spectrum of the LED is also shown in Figure 5.1. All spectra were corrected for dark current and stored in a file for later analysis.

Subjects

Three cohorts of healthy subjects were used in this study. The first group consisted of 61 subjects of Afro-Caribbean descent with a negroid dark skin color, living in the Netherlands. The second group was a group of 120 southern Chinese subjects with intermediate skin color, living in China. The third group consisted of 60 subjects of Asian and African descent, all living in the Netherlands. Health status was obtained by clinical assessment (first and second cohorts) or using a self-administered questionnaire (third cohort). For all these cohorts, only subjects with a UV-reflectance below 12% and with subject age between 20 and 70 years were included. Subjects were excluded if not all spectra were obtained correctly.

For assessing correlations between age-corrected skin *AF* and various parameters that were derived from reflectance spectra in the UV-A and visible range, a subset of 99 subjects (33 subjects from each cohort) was chosen from the total group. The

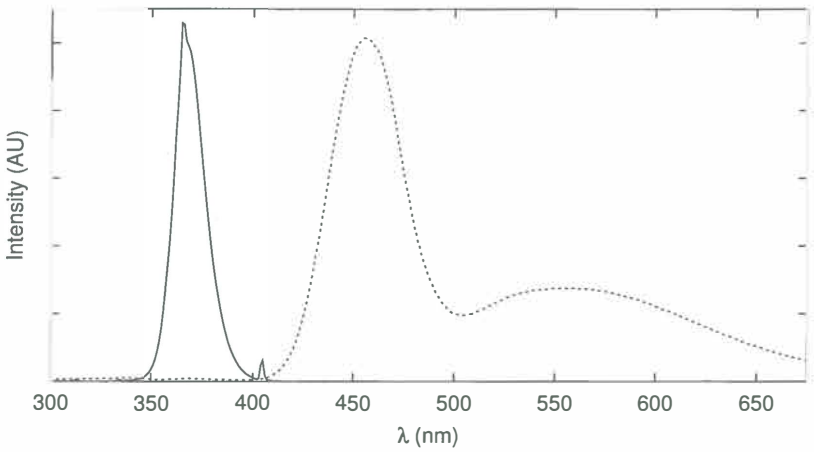

Figure 5.1: *Spectra of the UV blacklight tube (solid line) and the white LED (dashed line) as used in the AGE Reader. The LED spectrum was magnified to fit on the same scale as the spectrum of the UV tube.*

selection focused on obtaining a group of subjects over the full range of age (20 – 70 years) and UV-reflectance values (~3% – 12%), and was otherwise random. For the validation, all other subjects were used (n = 142).

Acquiring model parameters

The new algorithm has been based on a model that describes the deviation of the measured AF from an expected value. The expected AF of an individual can be described as a function of subject age in years, with $AF = 0.024\ age + 0.83$. This relation is based on a large set of Caucasian healthy persons with a UV-reflectance value above 10%.[1] With this, the deviation of skin AF for a particular individual is calculated as

$$\Delta AF = AF_m - AF(age) = AF_m - 0.024\ age - 0.83, \tag{5.1}$$

where AF_m is the skin AF as measured. ΔAF was used as the dependent variable in the fitting model.

The next step was finding parameters that describe the skin color and can be measured with the AGE Reader. For this, two types of spectra are available. First,

the spectrum that is measured directly from the skin during illumination with the UV light source. This spectrum includes a large peak of UV light that is reflected from the skin and a small emission peak, due to autofluorescence of the AGEs and possibly also other skin compounds with fluorescence emission in the same wavelenth region, such as NADH and lipofuscins. Secondly, a reflectance spectrum is available that represents the relative skin reflectance as compared to a white reference standard. This spectrum consists of two parts, one measured with the UV light source, ~350 – 410 nm, and one measured with a white light source, ~415 – 675 nm. The parameters were based on literature study and own observations.

It is not known whether the parameters as calculated from the spectra are independent of subject age. Therefore, also subject age was included in the model, to compensate for possible interactions.

The parameters were assessed for normality and collinearity using SPSS (version 16, SPSS Inc., Chicago, IL). Parameters were considered normally distributed if a Kolmogorov–Smirnov test resulted in a p-value above 0.05. Parameters were considered independent if the tolerance level exceeded 0.01. For the backward multivariate analysis, threshold p-values of 0.01 and 0.05 were considered.

Principle of the algorithm

With the parameters found, a prediction model for ΔAF was obtained, using a backward multiple linear regression analysis. Since the average expected ΔAF of any group of healthy subjects is assumed to be zero, the predicted ΔAF, ΔAF_{pred}, was then used as a correction for AF as

$$AF_{corr} = AF_m - \Delta AF_{pred}. \tag{5.2}$$

Validation

Since the low skin AF values were first observed in subjects that had a UV-reflectance below 10%, the derived algorithm for calculating skin AF can be validated by describing skin AF as a function of the UV-reflectance. For this, age-corrected skin AF, $\Delta AF_{corr} = AF_{corr} - AF(age)$, is used. Requirements are that ΔAF_{corr} should not be dependent on UV-reflectance and mean ΔAF_{corr} should be close to zero. Furthermore, the increase of skin AF values with subject age should match the reference values that were found earlier.[1]

Table 5.1: Group characteristics of the datasets used.

		UV-reflectance (%)		age (years)	
group[a]	size	mean ± sd	range	mean ± sd	range
Measurements used for model development					
group 1 (AC)	33	4.59 ± 1.36	2.55 – 7.99	41.5 ± 11.5	20 – 69
group 2 (SC)	33	9.16 ± 1.54	6.69 – 11.79	40.4 ± 15.8	21 – 70
group 3 (VO)	33	8.49 ± 2.21	4.13 – 11.53	40.9 ± 12.8	20 – 69
total	99	7.41 ± 2.66	2.55 – 11.79	40.9 ± 13.3	20 – 70
Measurements used for validation					
group 1 (AC)	27	5.32 ± 1.68	3.20 – 10.40	41.6 ± 13.5	20 – 70
group 2 (SC)	87	8.61 ± 1.84	4.30 – 11.55	46.8 ± 11.3	24 – 69
group 3 (VO)	27	9.03 ± 2.23	4.20 – 11.68	33.7 ± 10.4	20 – 58
total	141	8.06 ± 2.31	3.20 – 11.68	43.3 ± 12.6	20 – 70

[a] Groups consisted of Afro-Caribbean (AC) and southern Chinese (SC) subjects and subjects of various origin (VO).

Results

Subjects

Table 5.1 summarizes group size, skin color and age characteristics of the three cohorts separately and as a whole, for the model development group and the validation group separately. UV-reflectance was used as a measure of skin color. In the first group (subjects of Afro-Caribbean descent), one subject was excluded because of an artifact in one of the spectra.

Parameters for prediction of ΔAF

The parameters that may predict the deviation of AF from an expected value, ΔAF, are described below. Most parameters are related to melanin, hemoglobin or bilirubin, since these are the strongest absorbers in the skin. The parameters were analyzed for correlations using the dataset of 99 subjects. Table 5.2 summarizes the parameters analyzed.

Figures 5.2 and 5.3 show typical reflectance and emission spectra of three subjects with values of UV-reflectance of 4.4%, 8.0% and 11.4% respectively. The reflectance

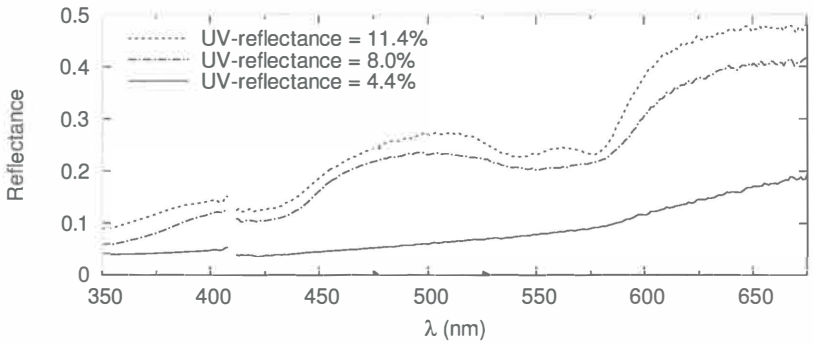

Figure 5.2: *Typical reflectance spectra of three subjects with varying values of UV-reflectance, showing different intensities of absorption bands. The gap around 410 nm is caused by insufficient intensity of the two light sources (blacklight tube and white LED) in that range. The apparent offset between the reflectance from both light sources is probably due to geometrical differences between the two light sources.*

spectra show some distinctive features, caused by absorption of melanin, hemoglobin and other chromophores. The intensity of these features varies between subjects and this information is used for the various parameters described below. As expected, the average reflectance of a subject with a dark skin color is lower. Figure 5.3 shows that the measured emission intensity is lower for subjects with darker skin color as well.

Reflectance in UV range

Since the start of the development of the AGE Reader, the UV-reflectance has been used as an indication of skin color. With this value, it was found that skin AF is lower than expected in subjects with darker skin colors.[10] No linear relation could be found with ΔAF. Some transformations were analyzed in order to arrive at a linear relation with ΔAF and the inverse value of the UV-reflectance (*InvRefl*) was linearly related to ΔAF. *InvRefl* is used as a parameter in the model, not the UV-reflectance itself.

Melanin related parameters

The amount of melanin may be expressed as an index. Sinichkin et al.[15] provided three wavelength ranges in which the ratio between the reflectance at two wavelengths (or the slope in a logarithmic spectrum) is used to determine this index.

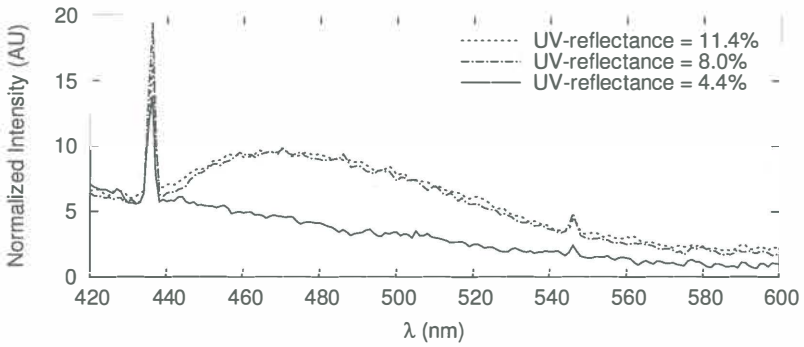

Figure 5.3: *Typical emission spectra of the same three subjects as shown in Figure 5.2. The spectra are normalized to the average intensity in the 300 – 420 nm region. The sharp peaks around 436 nm and 546 nm are caused by mercury emission from the UV light source. The subjects with 11.4% and 8.0% UV-reflectance have similar emission peaks, whereas the subject with 4.4% UV-reflectance has a lower emission peak.*

First, the UV-A wavelength is used, because of the high absorption of melanin in UV. The suggested wavelength range is from 365 – 395 nm. In the AGE Reader, the UV light source is illuminating in this range. However, in our measurements it was found that using 5 nm lower wavelengths yielded a better correlation with ΔAF. This wavelength range is centered around the peak wavelength of the light source used. The first melanin index (MI) parameter was defined as

$$MI1 = \frac{R_{390}}{R_{360}}, \tag{5.3}$$

where R is reflectance and the subscripts denote the wavelength in nm.

MI may also be derived from the near infrared region, where hemoglobin absorption is relatively small. Kollias and Baqer used wavelengths up to 720 nm.[16] However, the white light source in the AGE Reader does not allow for this range, therefore, wavelengths up to 675 nm were used. Since two references[16, 17] used different starting wavelengths, both pairs 620 / 675 nm and 650 / 675 nm were used in our study:

$$MI2 = 100\,(OD_{650} - OD_{675}) \tag{5.4}$$

$$MI3 = 100\,(OD_{620} - OD_{675}) \tag{5.5}$$

where OD_λ is the apparent optical density at wavelength λ, defined as $-log\ R_\lambda$.

Although Sinichkin et al. proposed these wavelength pairs as ratios in the *OD* spectrum, Kollias and Baqer used a regression through the spectrum instead. This method is less prone to artifacts because it does not rely on just two values in the reflectance spectrum. Therefore, we introduced another parameter, *RedLnSlope*, representing the slope of the regression line through the spectrum of $Ln(R)$ in the range 630 – 675 nm, multiplied by 100.

With more melanin, the melanin absorption causes a stronger decrease in the total reflectance spectrum, especially in the UV-range where melanin is the most important absorber. Figure 5.4 shows part of the normalized reflectance spectra as measured with the AGE Reader from healthy subjects with light and dark skin color. Both lines represent the average reflectance from six subjects, which were selected for having similar UV-reflectance values (approximately 18% for light skin color and approximately 6% for dark skin color). The shape of the spectrum of the subjects with light skin color appeared convex, whereas that of subjects with dark skin color showed to be concave. This shape can be quantified by assuming a line in the spectrum from the reflectance at 360 nm to the reflectance at 390 nm and then observing the deviation of the reflectance at 375 nm from the line. The shape was thus defined as

$$UVshape = \frac{R_{360} + R_{390}}{2\,R_{375}}. \tag{5.6}$$

No correlation was found between *UVshape* and ΔAF for subjects with darker skin colors ($R^2 = 0.077$). However, a linear correlation was found between *UVshape* and R_{390}, the reflectance at 390 nm, showing that *UVshape* is indeed dependent on skin color. With this correlation ($R^2 = 0.35$), a deviation was calculated per measurement, as a function of *UVshape* and R_{390}:

$$dUVshape = UVshape + 0.407\,R_{390} - 1.036. \tag{5.7}$$

This deviation value was found to correlate linearly with ΔAF and was used as a parameter.

Furthermore, absolute reflectance values may be correlated to ΔAF. In order to avoid interaction with hemoglobin, wavelengths had to be used where hemoglobin absorption is relatively low. Although no large differences in oxygen saturation were expected in healthy subjects, influence of oxygen saturation can easily be omitted by using isobestic points, where oxygenated and de-oxygenated hemoglobin have equal absorption.

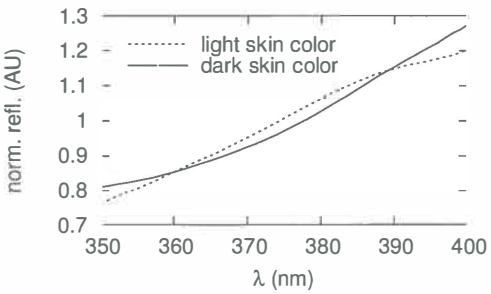

Figure 5.4: *Reflectance in the UV-A range (in arbitrary units, AU) of subjects with a light skin color and with a dark skin color. The lines represent the average reflectance of six healthy subjects with UV-reflectance of approximately 18% or 6% respectively. For comparison of the shapes, both spectra have been normalized such that average reflectance in the 350 – 400 nm range is 1. Subjects with a light skin color have a convex reflectance spectrum, subjects with a dark skin color have a concave reflectance spectrum.*

Reflectance at the hemoglobin absorption minimum and isobestic point around 500 nm was first assessed. A linear correlation with ΔAF was found after a logarithmic transformation. The transformed parameter is referred to as *LnR500*. Finally, *RedRefl* was introduced as the mean reflectance in the 620 – 675 nm range.

Hemoglobin related parameters

Erythema is a condition where the apparent influence of hemoglobin in the skin is increased. Sinichkin et al.[15] have summarized the mostly used parameters that assess erythema as an index (*EI*), using reflectance spectra. These indices can be used to describe the influence of hemoglobin on skin *AF* values. Two different methods to describe erythema have been used. The first was based on the area under the spectral curve of the apparent optical density in the 510 – 610 nm range, calculated as

$$EI1 = 100\,[OD_{560} + 1.5\,(OD_{545} + OD_{575}) - 2.0\,(OD_{510} + OD_{610})], \qquad (5.8)$$

where this wavelength range was chosen to include the specific hemoglobin absorption peaks.

The second method was a simplified version based on comparison of the reflectance at a wavelength where hemoglobin absorptivity is high (560 nm) and at

a wavelength where hemoglobin absorptivity is low (650 nm).[18] Erythema index was thus defined as

$$EI2 = 100\,(OD_{560} - OD_{650}). \tag{5.9}$$

Both parameters correlated linearly with ΔAF and were used in the model.

Although it was not expected that erythema should be different as a function of skin color, it was expected that a combination of erythema index as calculated with the two suggested methods would yield a good estimate of melanin influence, because the simplified *EI2* method ignores the contribution of melanin absorption, while the first method (*EI1*) should be independent of melanin absorption.

Furthermore, Feather et al.[19] developed formulas that describe hemoglobin concentration and oxygenation as indices, based on measurements at isobestic points. These indices were included in the model as parameters

$$HI = 100\left(\frac{OD_{544} - OD_{527.5}}{16.5} - \frac{OD_{573} - OD_{544}}{29}\right) \tag{5.10}$$

and

$$OI = \frac{5100}{HI} \times \left(\frac{OD_{573} - OD_{558.5}}{14.5} - \frac{OD_{558.5} - OD_{544}}{14.5}\right) + 42. \tag{5.11}$$

Bilirubin related parameters

Bilirubin has an absorption peak around 470 nm, which is within the emission range of the skin *AF* measurement, and has almost no absorption at 500 nm.[20] To assess the possible additional influence of bilirubin absorption, the ratio of the reflectance at 470 and 500 nm was included in the model as bilirubin index:

$$BI = \frac{R_{470}}{R_{500}}. \tag{5.12}$$

Emission related parameters

It was expected that besides the reflectance spectra, also the emission spectra contained information that could be correlated to ΔAF. Because absolute intensities are related to fluorophore content, only relative intensities can be used. Ratios of emission intensities at wavelength pairs 470 and 500 nm (*Em1*), 470 and 570 nm (*Em2*) as well as 600 and 650 nm (*Em3*) were included as parameters. The ratio between mean emission in the 470 – 500 nm and 600 – 650 nm ranges was included as parameter *Em4*.

Table 5.2: *Results from univariate linear correlations. For each parameter in the model, the square of Pearson's coefficient of correlation is presented (R^2). Normality is assessed using a one-sample Kolmogorov–Smirnov test. Values of p above 0.05 indicate a normal distribution.*

parameter	description[a]	R^2	normality (p)
InvRefl	Inverse of reflectance in UV range	0.452	< 0.01
MI1	Ratio of reflectance at 390 and 360 nm	0.701	0.27
MI2	Difference of *OD* at 650 and 675 nm	0.651	< 0.01
MI3	Difference of *OD* at 620 and 675 nm	0.676	< 0.01
RedLnSlope	Slope of line through *Ln* reflectance in 630 – 675 nm	0.681	< 0.01
dUVshape	Deviation of UV reflectance from straight line	0.541	0.65
LnR500	Natural logarithm of reflectance at 500 nm	0.638	< 0.01
RedRefl	Mean reflectance in 620 – 675 nm range	0.564	< 0.01
EI1	Area under curve of apparent *OD* spectrum in 510 – 610 nm range	0.202	0.65
EI2	Difference of *OD* at 560 and 650 nm	0.471	0.47
HI	Hb absorption measured at isobestic points	0.174	0.91
OI	Oxygenation index based on ratio single/double absorption peak Hb	0.001	0.04
BI	Ratio of reflectance at 470 and 500 nm	0.080	0.88
Em1	Ratio of emission at 470 and 500 nm	0.029	< 0.01
Em2	Ratio of emission at 470 and 570 nm	0.004	0.20
Em3	Ratio of emission at 600 and 650 nm	0.082	< 0.01
Em4	Ratio of emission in 470 – 500 and 600 – 650 nm ranges	0.115	< 0.01
Age	Subject age	0.105	0.60

[a] *OD* is defined as $-log$ reflectance.

Table 5.3: *Resulting parameters of the multiple regression analysis. The three-parameter model ($p < 0.01$ threshold) had an adjusted R^2 of 0.804.*

parameter	β	p	collinearity
(constant)		0.007	
MI1	0.406	0.000	0.226
RedLnSlope	-0.463	0.000	0.228
Age	-0.274	0.000	0.977

Univariate analyses

In the dataset of 99 subjects, the parameters as described above were assessed for linear correlation with age-corrected AF, ΔAF. Table 5.2 summarizes the univariate linear correlation coefficients (expressed as Pearson's R^2) that were found for correlations between ΔAF and the various parameters. Because all parameters were designed or transformed as such, only linear correlations existed. Normality was assessed using the Kolmogorov–Smirnov test for each parameter. Significance values of normality (p) are also shown in Table 5.2. It should be noted that not all parameters had a normal distribution.

Acquirement of the new algorithm

The parameters as described above were used in a backward multiple linear regression analysis to find a model to describe ΔAF. When a $p = 0.05$ threshold was used, four parameters contributed (*dUVshape* and the three parameters as listed in Table 5.3). The parameter with the lowest relative contribution, *dUVshape*, had a β value less than half of that of the *MI1* and *RedLnSlope* parameters. Herein, the standardized correlation coefficient β represents the contribution of a specific parameter relative to the contribution of others. Adjusted R^2 was 0.814, not different from the adjusted R^2 level of 0.804 with the three-parameter model with a $p < 0.01$ threshold level, which is shown in Table 5.3. If subject age was not included, adjusted R^2 was 0.731. ΔAF can thus be described as a linear combination of the parameters in Table 5.3, and the new algorithm for calculating skin AF has been based on these parameters.

Collinearity was assessed as well. Although collinearity was found between some parameters, the significant parameters in the model are independent (tolerance above 0.01).

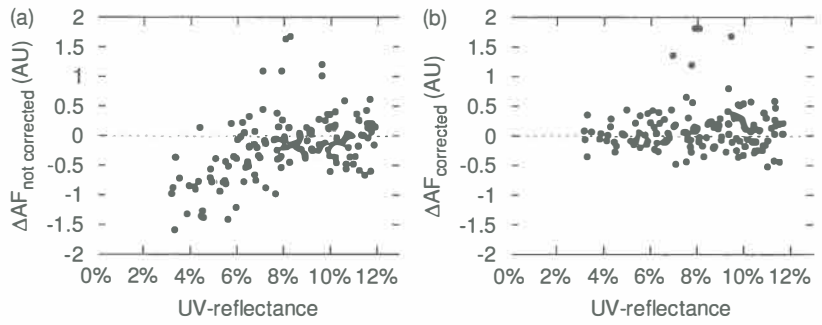

*Figure 5.5: Age-adjusted skin AF (ΔAF) as a function of UV-reflectance as calculated without correction for skin color (**a**) and with the new algorithm (**b**).*

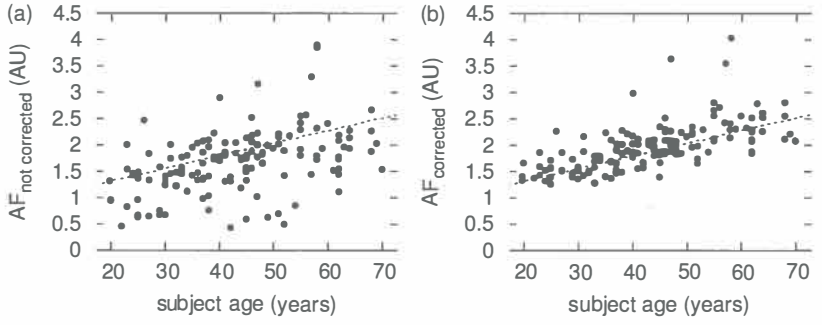

*Figure 5.6: AF values as a function of subject age as calculated without correction for skin color (**a**) and with the new algorithm (**b**). The dashed line represents the reference values for Caucasian subjects, $AF = 0.024\ age + 0.83$.*[1]

Validation

Using the algorithm as obtained above, the corrected value of skin AF, AF_{corr}, was calculated using

$$AF_{corr} = AF_m + \alpha_1 \, MI1 + \alpha_2 \, RedLnSlope + \alpha_3 \, Age, \tag{5.13}$$

where AF_m is the measured uncorrected skin AF, and α_1 through α_3 are multiplication constants that were derived using the multiple regression analysis. Skin AF (AF_{corr}) and age-adjusted skin AF (ΔAF_{corr}) were calculated for each individual in the validation-group. ΔAF_{corr} was calculated using Equation 5.1, using AF_{corr} instead of AF_m. This group consisted of 27 subjects from the Afro-Caribbean cohort, 87 subjects from the South Chinese cohort and 27 from the cohort of subjects of various origin. Age-adjusted skin AF is shown as a function of UV-reflectance values in Figure 5.5, also comparing the new algorithm (b) and the old method for calculating skin AF (a). With the new algorithm, the mean standard deviation of ΔAF_{corr} as percentage of the skin AF is 14.8%.

Figure 5.6 shows the AF values as a function of subject age, as calculated with the new algorithm (b) as well as without correction for skin color (a). Values are compared with the standard reference line as obtained from Caucasian subjects.[1]

Discussion

The current study describes and validates a new calculation algorithm for skin AF that enables reliable determination of increased skin AF in subjects, regardless of the color of the skin. For this algorithm, parameters were applied that were derived from reflectance spectra as measured on the skin that correlate with the originally observed decrease in skin AF values of subjects with a dark skin color.

To compensate for differences in skin color, skin AF was initially calculated as the mean light intensity in the emission range divided by the mean light intensity of the light that is reflected from tissue in the excitation range, as suggested previously by Coremans et al.[21] Whenever more melanin or other skin compounds are absorbing emission light, they also absorb more excitation light and by dividing these two quantities, the result will be less dependent on absorption. Using this method, skin AF can reliably be obtained in subjects with Fitzpatrick skin phototypes I – IV. Stamatas et al.[22] also used the reflectance of the skin as normalization factor for autofluorescence measurements. They also reported that this method is adequate, but

only for lighter skin types. In the AGE Reader, a simple skin color assessment is performed using the mean intensity of the UV-A light that is reflected from the skin. It was found that skin *AF* can be reliably assessed if more than 10% of the UV-A light is reflected.[1, 10] This method could not compensate for the strong absorption of melanin, as in subjects with a dark skin color.

In the AGE Reader, the excitation light source illuminates in the 350 – 410 nm range and emission is measured in the 420 – 600 nm range. Skin *AF* in these ranges may not only be caused by skin AGEs. Also other fluorophores such as keratin, vitamin D, lipofuscin, ceroid, NADH and pyridoxine may add to the total fluorescence signal.[23] Furthermore, some fluorophores have excitation maxima that are within the emission range of the fluorophores above, including porphyrins, elastin crosslinks, FAD, flavins and phospholipids. Due to the overlapping nature of absorption and emission spectra, it is difficult, if not impossible, to assess the influence of specific fluorophores on the total fluorescence signal, especially with the broad excitation peak that is used in the AGE Reader. However, it has been shown that even with this broad excitation peak, dermal content of specific AGEs explains the major part of the variance (up to 76%) in the skin *AF* signal in a pooled analysis of the validation studies mentioned earlier,[2–4] and, moreover, that the risk of chronic complications in diabetes can be assessed.[24]

Apart from other fluorophores, non-fluorescent chromophores in the skin may have an effect on skin *AF* by selectively absorbing excitation and/or emission light. The most contributory chromophores in the UV-A and visible region are melanin in the epidermis and hemoglobin in the dermis.[15, 20, 25] Both in the epidermis and the dermis, also bilirubin and to a lesser extent beta-carotene are present, having absorption peaks at 470 nm and 450 nm respectively.[20, 23] Nevertheless, melanin and hemoglobin are widely accepted as the main absorbers.

The absorption spectrum of melanin has been studied extensively in vitro.[26] However, melanin resides in the skin in cell organelles, melanosomes, and the effect on skin color and moreover on the measurement of *AF* is influenced by the size, number, distribution and aggregation of these melanosomes in the skin, which may vary largely between individuals of different ethnic groups.[27, 28] In general, melanin absorbs light from the UV, visible and near infrared range of the spectrum, with an exponential increase of absorption towards lower wavelengths.[26, 29]

Hemoglobin has a broad absorption spectrum over the visual part of the spectrum with several absorption peaks and is therefore an important factor in skin color.[19, 20, 23] Although it is not expected that the hemoglobin concentration or distribution is very

different for the various skin phototypes, the apparent optical properties of hemoglobin and their influence on skin AF may vary because of interactions with other chromophores (e.g. melanin) during light propagation within the skin. Moreover, hemoglobin is concentrated in red blood cells within blood vessels. Because of a limited and wavelength dependent penetration depth of light in blood vessels, the influence of hemoglobin on skin AF is difficult to assess. Nevertheless, Na et al. observed a variation of skin autofluorescence in their measurements as a function of skin redness, which depends on hemoglobin concentration or oxygen saturation.[30]

Several approaches exist to describe the influence of absorbers and scatterers on skin color. Some methods have used a homogeneous approach,[11–15] whereas others have defined many layers in the skin, with separate optical properties in each layer, that may vary between subjects.[31–34] Some of these approaches aim at determining the concentration of certain chromophores or identifying specific fluorophores. Since several questions still remain to be solved, the current study has chosen for a more practical approach by using the spectra that are measured individually in the UV-A and visible range (350 – 675 nm), focusing on developing an algorithm to correct skin AF in healthy subjects for the influence of skin color, using parameters that are derived from these spectra. For this correction, the main characteristics of only the strongest contributing absorbers, melanin, hemoglobin and bilirubin, have been used as a basis for finding significant spectral properties that may describe the lower skin AF values in subjects with a dark skin color. To achieve a simple model, influence of specific fluorophores and less contributory absorbers have not been taken into account.

The developed model, using subject age and two parameters from the reflectance spectrum, could account for over 80% of the relative change in skin AF values. The new calculation algorithm, based on this model, yields skin AF values that are almost independent of skin color, even without knowing the exact composition of chromophores, fluorophores and scattering particles in the skin.

If a 0.05 threshold was used, the additional *dUVshape* parameter would be included, which had a β value of less than half of that of the other two parameters, *MI1* and *RedLnSlope*. Adjusted R^2 was not better than for the preferred model with only two spectral parameters. Therefore, in this study, the low threshold of 0.01 was chosen for excluding parameters from the model.

In the current study, only age and the *MI1* and *RedLnSlope* parameters of Table 5.2 were necessary to describe the influence of skin color on skin AF. All other parameters, including the parameters from the emission spectra, could be discarded from the

final model. A bilirubin related parameter was studied as well because we initially assumed that small changes might also influence the measured skin AF. Nevertheless, it should be noted that a significant influence on skin AF may be present in conditions such as jaundice. Similarly, the present results can not exclude that strong erythema may also influence skin AF.

Subject age is an important predictor for skin AF values. Therefore, an age-corrected value, based on reference values of skin AF in Caucasian subjects,[1] was used in the model. However, the age-dependence of skin AF may be different depending on racial or cultural differences, e.g. dietary variations or smoking habits. By applying the same relation of skin AF and age to all subjects, equal reference values can be used, allowing the detection of increased skin AF independent on skin color. Our results show that corrected AF has the same increase with subject age for the entire group of subjects from various descent.

Figure 5.5 shows some subjects that have higher skin AF values as compared to other subjects of the same age, even after correction (ΔAF value above 1). We assume that these subjects may have developed an increased cardiovascular risk, without immediate clinical symptoms. It should be noted that in the cohort that was used for developing the model, no increased values of skin AF were observed (not shown).

The inclusion of subject age in the model may seem unnecessary at first, because the model was designed to predict ΔAF, which reflects a value independent of age. However, it was assumed that age could have an effect on other parameters. Although age did not correlate to any of the parameters, it turned out to be a significant predictor in the model. If age was left out from the model, adjusted R^2 decreased to 0.731.

Although we did not yet attempt to physically explain our observations, the current study suggests that for the purpose of assessing skin AGEs, the influence of skin color on the AF measurements may be sufficiently described using age and the *MI1* and *RedLnSlope* parameters, i.e. the ratio of two reflectance values in the 360 – 390 nm range and the slope of the reflectance in the 620 – 675 nm range. This resulted in a mean standard deviation of 14.8% of the AF values, which is even lower than the 20% that was observed in a Caucasian group from an earlier study.[1] Therefore, we may have successfully developed a technique to recognize increased values of skin AF independent of skin color.

In conclusion, an algorithm to calculate skin AF was developed and validated for subjects between 20 and 70 years. With this new method, skin AF can now be measured independent of skin color, which makes the measurement of skin AF for the non-invasive assessment of increased levels of skin AGEs more generally applicable.

Acknowledgements

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Competing interest

R. Graaff and A.J. Smit are also founders and stockholders of the university spinoff DiagnOptics Technologies B.V., manufacturer of the AGE Reader (www.diagnoptics.com). M. Koetsier is a Ph.D. student at the Department of Biomedical Engineering whose study is partly financed by DiagnOptics Technologies B.V.

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CHAPTER 6

Optical skin spectra reflect changes in tissue after hemodialysis

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Submitted

Abstract

Skin color is known to change with differences in the state of patients. A single hemodialysis treatment provides a good model for studying this effect because it causes the removal of uremic toxins and changes in hematocrit and tissue oxygenation within a few hours. We hypothesized that examination of optical spectra as measured from the skin before and after a hemodialysis session may provide information about the effectiveness of this treatment. In this study, measured skin autofluorescence and reflectance spectra were systematically analyzed for changes. These changes could be correlated to a decrease of plasma fluorescence, as a marker of hemodialysis effectiveness. Total skin autofluorescence, a marker of accumulation of advanced glycation end products, did not change after hemodialysis. It is concluded that emission and reflectance spectra have the potential to provide information about the metabolic state of hemodialysis patients and can be used to monitor these patients.

Introduction

The color of the skin is an important clinical assessment tool for metabolic changes. For instance, end-stage renal disease (ESRD) has an effect on the color of the skin, e.g. by hyper-pigmentation.[1] It has been suggested that these color changes are the result of accumulation of compounds in the skin that would otherwise have been excreted by the kidneys.[2] Hemodialysis partly replaces the renal function, removing water and accumulated water soluble compounds from the plasma. This results in changes in hematocrit and blood oxygenation, causing changes in the apparent light absorption by hemoglobin.[3] Therefore, we hypothesize that examination of changes in skin color may provide information on metabolic changes, such as found after regular hemodialysis treatment.

To describe the color of the skin in this respect, two methods are reported. First, color can be described as values of melanin index (MI) and hemoglobin index (EI), which are parameters derived from reflectance measurements at 568 nm, 660 nm and 870 or 880 nm wavelengths.[4,5] Second, color can be described in the L*a*b* system of the Commission Internationale de l'Éclairage (CIE).[6] In this system, the L* value has been shown to relate to the MI value and the a* value to the EI value.[7] Both methods have the advantage that the color is simply described in a few terms, which facilitates easy comparison. However, a drawback is that most optical information is lost. With the MI and EI system only differences at three wavelengths are observed. The three values in the CIE L*a*b* system can often be the result of several distinct spectra and this system only involves the visual part of the spectrum.

Another optical measurement of the skin is that of skin autofluorescence (*AF*).[8] Measuring skin *AF* is a non-invasive method for assessing the amount of accumulated tissue Advanced Glycation Endproducts (AGEs) and can be calculated from the mean emission in the 420 – 600 nm range normalized by the amount of UV-A excitation with a peak wavelength of 370 nm. A significant correlation exists between skin *AF* and levels of several AGEs.[9] Skin *AF* has shown to increase with age[10] and is an important marker in cardiovascular risk assessment.[11–16] However, fluorescent AGEs and other fluorophores also reside within the bloodstream and it is unknown to what extent this influences the value of skin *AF*. The amount of fluorescence within the blood might fluctuate in several physiological conditions, possibly confounding the assessment of tissue AGE accumulation.

In an earlier study, we presented several characteristics of diffuse reflectance spectra and emission spectra that describe the influence of skin color on the measurement

of skin AF.[17] In the current study, these characteristics were used in a broader sense to describe skin color. In the present study, a systematic search was performed to find additional characteristics by observing changes in the skin spectra that result from the hemodialysis session. Because part of the compounds that are removed during hemodialysis are fluorescent, the effectiveness of the hemodialysis treatment was assessed by the decrease of plasma fluorescence.

The aim of the current study was to describe the changes in the emission and diffuse reflectance spectra of the skin and skin AF as a result of a single hemodialysis session. Spectral differences were correlated to the reduction of plasma fluorophores, as a marker of hemodialysis effectiveness.

Results

Skin autofluorescence and plasma fluorescence

Total skin AF in the 420 – 600 nm range did not change after the hemodialysis session ($p = 0.53$). In contrast, changes in the shape of the emission spectra per nm did occur, as described in more detail below. Fluorescence of plasma samples decreased 20% after the session ($p < 0.001$) for 330/380 nm fluorescence (excitation/emission) and 12% ($p < 0.001$) for 370/460 nm fluorescence.

Skin reflectance

Reflectance spectra were obtained in the 350 – 675 nm range. Reflectance spectra changed during the hemodialysis sessions in several ways, as described in more detail below. As an example, Figure 6.1 shows the reflectance spectra of one patient as measured before and after the hemodialysis session.

Describing changes in spectra as spectrum characteristics

A systematic search was performed to find changes in the skin emission and reflectance spectra that may correlate to the decrease in plasma fluorescence (PF). This decrease was expressed as the ratio of plasma fluorescence after hemodialysis divided by the plasma fluorescence before (ratio of PF, RPF). The absolute change in skin fluorescence emission intensity per nm correlated to RPF in the 410 – 435 nm range. Furthermore, relative changes were assessed, defined as the change in the

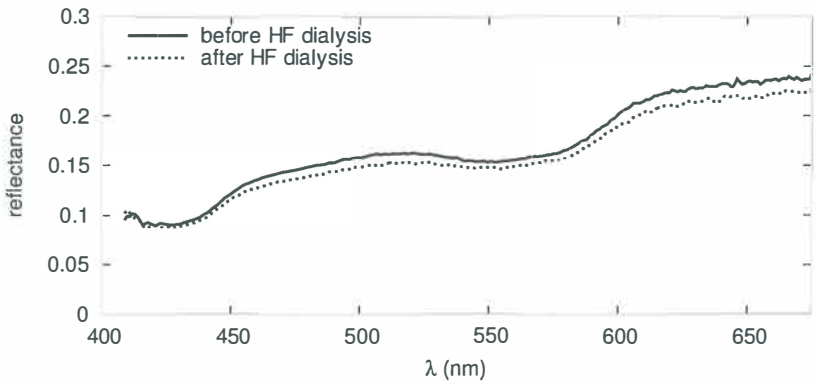

Figure 6.1: *Diffuse skin reflectance spectra of one patient as measured before and after the hemodialysis session, in the 409 – 675 nm range, as measured with the white LED. At the lower wavelengths, the reflectance is almost equal. At higher wavelengths, the reflectance is lower after the hemodialysis session.*

intensity at one wavelength as compared to the intensity at another wavelength. In this assessment, all wavelength pairs, per nm, in the 350 – 675 nm range were used. The diffuse reflectance spectra and the fluorescence emission spectra of the skin were assessed separately. For each wavelength pair, the change in the spectrum was tested for correlation with *RPF*. Surface-plots were generated of the p-values from these Spearman tests as a function of the wavelength pairs. Figure 6.2, an example of these plots, clearly shows some areas (indicated by the arrows) where correlation occurred between the spectral changes and *RPF*. From such areas, 12 more spectrum characteristics were defined for the further analysis.

Changes after hemodialysis

The spectrum characteristics that changed significantly after one hemodialysis session (Wilcoxon signed-rank test, $p < 0.05$) are listed in Table 6.1, together with details on how they were calculated. Since no correction was performed for multiple statistical comparison of the dataset, this analysis only indicated where changes in the spectra may be observed. In the listed spectrum characteristics *RedLnSlope*, *MI3* and *RedSlope*, all calculated from the 620 – 675 nm range of the spectra, reflectance decreased for the lower wavelengths as compared to the higher wavelengths. The same effect was seen in the *n06* and *n10* spectrum characteristics, as calculated from

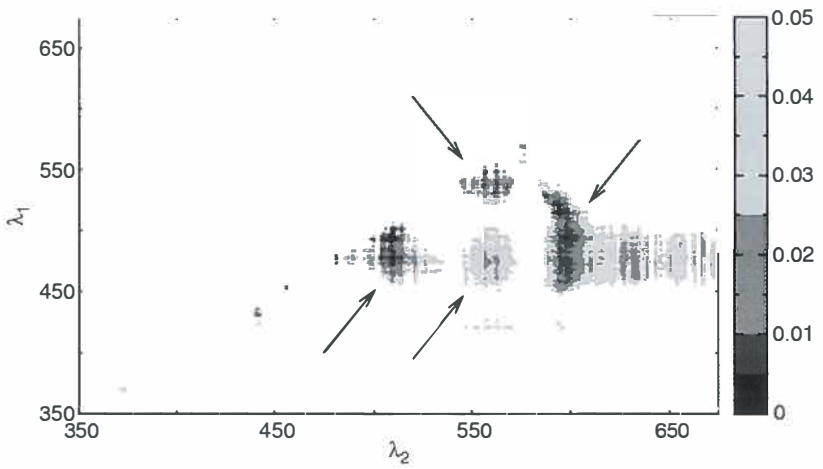

Figure 6.2: *Spearman's significance (p, gray-value in the plot) of the correlations for changes in skin reflectance and RPF, as an example of the analysis. The four arrows point at regions of interest that were assessed. See the main text for a further description.*

the 550 – 605 nm range. The increased values of the *Em2* characteristic indicate a slightly stronger emission at 470 nm as compared to the emission at 570 nm. The increased values of the *n12* characteristic suggest more reflectance around 395 nm as compared to the reflectance around 585 nm. Finally, the higher values of the *UVshape* characteristic suggest a lower reflectance at 375 nm as compared to the reflectance at 360 and 390 nm.

Linear multivariate modeling of the change in plasma fluorescence

The changes in the skin spectrum characteristics after the hemodialysis session, subject age and gender and the change in skin *AF* were used as variables in a backward multiple regression analysis to describe *RPF*. The change in 330/380 nm (excitation/emission wavelengths) *PF* was explained for 71% (adjusted R^2) using changes in 8 spectrum characteristics and the gender of the subjects, as listed in Table 6.2 as model *A*. The change in 370/460 nm *PF* (model *B*) was explained for 40% (adjusted R^2) using changes in 5 spectrum characteristics. Table 6.2 lists the variables that were used in these two models, together with what these characteristics represent.

Table 6.1: *Most significant changes (Wilcoxon signed-rank test, $p < 0.05$) of skin spectrum characteristics (sp. char.) after one hemodialysis session and how they were calculated. The spectrum characteristics n06, n10 and n12 were derived in the current research, the others were presented in earlier work.*[17] *The ratio was calculated as the value after hemodialysis divided by the value before hemodialysis.*

sp. char.	ratio	p	description
Em2	1.0203	0.047	Intensity of the emission light at 470 nm divided by the emission at 570 nm.
RedLnSl	1.0898	0.028	Slope of the natural logarithm of the reflectance spectrum in the 630 – 675 nm (red) region.
UVshape	1.0020	0.003	Shape of the reflectance spectrum in the UV-range, calculated from the reflectance at 360, 375 and 390 nm.
RedSlope	1.0077	0.021	The ratio of reflectance at 620 and 675 nm.
MI3	1.1319	0.021	Melanin Index, calculated from the reflectance at 620 and 675 nm.
OI	0.7073	0.038	Hemoglobin Oxygenation Index, based on the ratio of the single and double absorption peaks of oxygenated and de-oxygenated hemoglobin between 500 and 600 nm.
n06	0.9880	0.010	Mean reflectance in the 450 – 570 nm range, divided by the mean reflectance in the 575 – 585 nm range.
n10	0.8941	0.038	Mean reflectance in the 585 – 605 nm range, subtracted from the mean reflectance in the 570 – 590 nm range.
n12	1.0377	0.016	Mean reflectance in the 390 – 400 nm range, divided by the mean reflectance in the 580 – 590 nm range.

Table 6.2: *Descriptions of the skin spectrum characteristics (sp. char.) that were used in the models that describe the ratio of measured PF after and before the hemodialysis session. Model A describes the change in 330/380 (excitation/emission) PF, model B describes the change in 370/460 PF. Model A also used the gender of the subject as a variable. The spectrum characteristics n02, n05, n08 and n15 were derived in the current research, the others were presented in earlier work.*[17]

sp. char.	model	description
Refl	*B*	Mean reflectance in the 300–420 nm range, calculated as the intensity as measured from the patient divided by the intensity as measured from a white reference standard.
InvRefl	*A*	Inverse of the Refl spectrum characteristic (1 / Refl).
BI	*A*	Bilirubin Index, calculated as the reflectance at 470 nm divided by the reflectance at 500 nm.
RedSlope	*A*	See Table 1.
MI2	*A*	Melanin Index, calculated from the reflectance at 650 and 675 nm.
HbI	*B*	Hemoglobin Index, based on the ratio of the single and double absorption peaks of oxygenated and de-oxygenated hemoglobin between 500 and 600 nm.
OI	*A*	See Table 1.
n02	*A*	Mean reflectance in the 370–390 nm range, subtracted from the mean reflectance in the 365–380 nm range.
n05	*A* and *B*	Mean reflectance in the 365–370 nm range, divided by the mean reflectance in the 372–385 nm range.
n08	*B*	Mean reflectance in the 550–565 nm range, subtracted from the mean reflectance in the 470–485 nm range.
n15	*A* and *B*	Mean reflectance in the 455–530 nm range, divided by the mean reflectance in the 590–605 nm range.

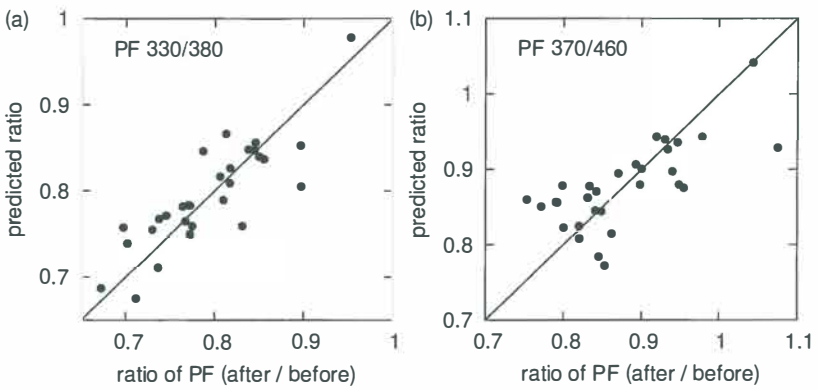

Figure 6.3: *The results of the describing models for the change in 330/380 nm (**a**) and 370/460 nm (**b**) PF. The vertical axis represents the predicted ratio of PF after and before the hemodialysis session as calculated with the model. The horizontal axis shows the measured ratio. The line represents the ideal model ($y = x$). R^2 of the regression lines through the data points (not shown) are 0.80 and 0.51 respectively.*

Figure 6.3 shows the predicted change in *PF* as calculated with the models as a function of the measured change, for both models separately.

Discussion

The current study investigated the possibility of assessing the metabolic change of a patient after a single hemodialysis session using diffuse reflectance and fluorescence spectra as measured from the skin. Changes in skin *AF* were assessed as well. With the observed changes, we could partly describe the decrease in plasma fluorescence as a function of the changes in spectrum characteristics.

The analysis of changes in skin spectra as a marker of change in metabolic state may seem a daunting task, resulting in a plethora of very small changes, making it impossible to generalize changes in spectra in certain clinical conditions. To solve this problem, several authors have described changes in the spectra using only two or three numerical variables.[4–6] Similarly, we have used several characteristics of skin reflectance and emission spectra to describe the influence of skin color on the measurement of skin *AF*.[17] In the current study, these characteristics were used in a broader sense to describe changes in skin color after a hemodialysis session.

Additionally, we have performed a systematic search for changes in the reflectance and emission spectra in the full 350 – 675 nm range in the current study. This resulted in 13 new spectrum characteristics that changed significantly after the hemodialysis session or correlated to the decrease of PF.

In the current clinical practice, the dose of a hemodialysis session is often assessed by the clearance of urea, expressed as either Kt/V or urea reduction ratio.[18] Additionally, interest has also been developed in the amount of AGEs that are removed from serum during hemodialysis.[19–21] The fluorescent properties of the AGE pentosidine has been used in this study to assess the clearance of AGEs during the hemodialysis and as a general measure of dialysis dose.

One spectrum characteristic that was specifically designed to represent the amount of hemoglobin absorption, the hemoglobin index[22] in Table 6.2, did not significantly change after hemodialysis. Moreover, erythema index, designed to describe the apparent influence of hemoglobin in the skin, calculated in two variations,[17] was not different after the hemodialysis session. We initially hypothesized that changes in the reflectance spectrum might partly be related to changes in hematocrit, which increases during hemodialysis because of the removal of fluid from the plasma. The light that is used for the measurement of the spectrum may therefore encounter more hemoglobin on its path through the tissue, resulting in a lower reflectance, especially in the strong absorption bands of hemoglobin (390 – 450 nm and 500 – 600 nm). On the other hand, the absorption from blood has shown to be related to the surface area of the blood vessels.[23] The removal of fluid by hemodialysis reduces the total blood volume and it is as yet unknown how this may influence the total surface area of the blood vessels and therewith the reflectance. It is therefore unclear how changes in hematocrit influence the measured skin spectra. Overall, no effects of possible hematocrit changes influencing the skin spectra were found in the present study.

Remarkably, the hemoglobin oxygenation index (OI) spectrum characteristic was 29% lower after the hemodialysis session. At first glance, enhanced oxygen supply to the tissue was expected after hemodialysis because of the higher hematocrit. Clinically, our results confirm an increased oxygen consumption as was reported during hemodialysis for muscular tissue, as obtained by spectrometry as well.[24] However, because the spectra we measured originate from the skin, that study may not fully explain our results. It is also possible that the OI characteristic, as measured by us, is not sufficiently representing the actual oxygenation of the tissue.

Three characteristics were calculated from the 620 – 675 nm range of the spectra: *RedLnSlope*, *MI3* and *RedSlope*. Reflectance decreased in this region and more so

for lower wavelengths within the region. Most of these characteristics were designed to detect absorption by melanin.[17, 25, 26] For wavelengths between 550 and 605 nm, the results also showed a stronger decrease in reflectance for lower wavelengths in that region, represented by the *n06* and *n10* spectrum characteristics. Apart from absorption by hemoglobin, this may also indicate an increased amount of light absorption by melanin. Interestingly, Murakami et al. found that pheomelanin is present in increased amounts in the blood of hemodialysis patients and they suggested that it is accumulated in the tissue of hemodialysis patients, partly because it is not dialyzed at all during hemodialysis due to its high molecular weight when bound to proteins.[27] Therefore, we did not expect that changes in the amount of melanin play a significant role in the spectral changes that we observed in the few hours of one hemodialysis session. The cause of the observed changes should be investigated further. Possibly, some other small and middle molecules exist that absorb light at these wavelengths and are removed during hemodialysis. Furthermore, the role of absorption by hemoglobin may play a role. Finally, reduced edema may increase the amount of melanin per unit area.

Besides the apparent changes in spectrum characteristics after one hemodialysis session, we have also attempted to describe the decrease in *PF* as a linear function of the changes in these spectrum characteristics. Remarkably, the largest and most significant changes in skin spectrum characteristics after one treatment session were not necessarily correlated best to *RPF*. Therefore, changes in all characteristics that showed a sufficient univariate correlation with *RPF* (Spearman's $r > 0.2$) were entered as variables in the model. This resulted in models that described the decrease in 330/380 (excitation/emission) fluorescence and 370/460 fluorescence for 71% and 40% respectively. Although it is not known exactly how the changes in the separate spectrum characteristics are related to changes in the amounts of the various molecules, these results strongly suggest that the use of diffuse optical techniques may aid in the assessment of changes in the metabolic state of a patient.

Finally, although our results showed significant changes in the amount of fluorescence of blood plasma, no changes in skin *AF* occurred. A reduction of skin *AF* after the session was expected because of the removal of AGEs from the blood. The fact that skin *AF* remains similar after the treatment may partly be due to the consumption of a meal during the hemodialysis. Noordzij et al. showed a significant increase of skin *AF* two to four hours after an AGE-rich meal.[28] They could not attribute this rise to meal-induced glycemic changes and suggested that skin *AF* was directly influenced by the AGE content of the meal. However, it has been suggested

before that AGE levels in plasma do not reflect tissue AGE levels,[29] although those authors have measured the tissue AGE levels in biopsies, i.e. separated from plasma, whereas we and also Noordzij et al. have measured skin *AF* in vivo. Our results suggest that skin *AF* is not influenced by *PF*, even if *PF* is considered with the same excitation and emission maxima as used for the measurement of skin *AF* (370 nm excitation and 460 nm emission).

In conclusion, we showed that diffuse reflectance spectra changed in various ways as a result of a single hemodialysis session. Skin *AF* did not change after hemodialysis and was not related to *PF*. Using changes in the skin spectra it was possible to partly describe the decrease in *PF* as an indication of hemodialysis effectiveness. This study attracts attention to the importance of utilizing non-invasive optical measurements of the skin in assessing metabolic diseases.

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Author contributions

M.K. and R.G. designed and executed the analysis methods. M.K. wrote the main text of the paper. B.S. and R.G. designed the clinical measurements. R.G., S.A., G.E. and B.S. performed the measurements. All authors discussed the methods, results and implications and commented on the manuscript.

Methods

Subjects

Thirty ESRD patients from the dialysis center of the University Hospital of Northern Sweden, Umeå, Sweden, were included in this European study. The study was approved by the Ethical Committee of Umeå. Subject age was 67 ± 12 years. The group consisted of 8 female and 22 male patients. Session duration was 12.6 ± 2.7

hours per week and 4.1 ± 0.6 hours for one session. Dialysis vintage was 36.6 ± 26.9 months, ranging from 5 to 110 months (values are mean ± 1 s.d.).

Measurement of skin autofluorescence and reflectance

Skin *AF* and diffuse reflectance spectra were measured on the volar forearm skin with the AGE Reader (DiagnOptics Technologies B.V., Groningen, The Netherlands), immediately before and after the hemodialysis session. A UV-A blacklight tube was used as illumination source. A non-contact optical fiber detected the emission and reflected excitation light at an angle of 45°. Using a spectrometer and computer software, the intensity spectrum was analyzed. The value of skin *AF* (in arbitrary units) was calculated as the ratio of the total emission intensity (420 – 600 nm) and the total excitation intensity (300 – 420 nm), multiplied by 100. The value was then corrected for the influence of skin color.[17] For the present study, the software had been adapted to also measure a complete diffuse reflectance spectrum in the UV-A and visible range (350 – 675 nm). A white Light Emitting Diode (LED) was used as illumination source in the visible range. Reflectance per nanometer wavelength interval was calculated as the measured intensity, divided by the intensity as measured from a white reference standard. Mean values of three successive measurements were used for the analysis. The change of skin *AF* after the hemodialysis session was assessed with a paired, two-tailed Student's t-test.

Measurement of plasma fluorescence (*PF*)

Total plasma fluorescence (*PF*) was measured according to a protocol of Schwedler et al.[21] In brief, blood samples were taken before and after the hemodialysis session and centrifuged and frozen at −80 °C for later analysis. For the analysis, the samples were thawed and diluted 50 times in phosphate buffered saline. Fluorescence was measured at 380 nm (upon excitation at 330 nm), and 460 nm (upon excitation at 370 nm) with a FLUOstar Optima plate-reader (BMG Lab Technologies, Durham, NC, USA). The change of *PF* after the hemodialysis session was assessed with a paired, two-tailed Student's t-test.

Describing changes in spectra as spectrum characteristics

The reflectance spectra were systematically assessed to find spectral characteristics that changed after the hemodialysis session or could be correlated to the change in

PF. The ratio of *PF* (*RPF*) was used for this, defined as the amount of *PF* after the hemodialysis divided by the amount before. *RPF* was calculated separately for 330/380 nm *PF* and 370/460 nm *PF*. This analysis was described in detail in the supplementary material.

Besides the spectrum characteristics as found in the systematical assessment, some spectrum characteristics were included in this study that were presented earlier to describe the influence of skin color on skin *AF*.[17] The characteristics from that work included slopes and numerical representations of the shape of the reflectance and emission spectra in certain wavelength regions. In the present study, changes in the measured skin spectra were described in terms of changes in all these spectrum characteristics. The ratios of the values after and before hemodialysis were assessed using the Wilcoxon signed-rank test ($p < 0.05$). Since no correction was performed for multiple comparison, results from these tests are only an indication of where in the spectra changes may be observed.

Linear modeling of the change in plasma fluorescence

For all spectrum characteristics, the changes after the hemodialysis session were tested for having a linear relation with *RPF*, using Pearson's linear regression tests. Kolmogorov–Smirnov (K–S) tests were used to assess normality ($p > 0.1$) instead of Shapiro–Wilk tests to avoid the strong influence of outliers. Co-linearity was assessed between all spectrum characteristics using Pearson's regression ($R^2 > 0.9$). Wherever co-linearity occurred, one characteristic was selected, based on the best univariate correlation (highest R^2) with *RPF*.

With the changes in the spectrum characteristics and skin *AF*, supplemented with subject age and gender, multivariate linear regression was performed to describe *RPF*. The backward regression method was used (inclusion: Pearson $R^2 > 0.04$, exclusion: $p > 0.05$). Separate analyses were performed for both *PF* wavelength pairs.

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Supplementary methods

Supplementary methods description accompanying *Optical skin spectra reflect changes in tissue after hemodialysis* by M. Koetsier et al.

Describing changes in spectra as spectrum characteristics

The reflectance spectra were systematically assessed to find spectral characteristics that changed after the hemodialysis session or could be correlated to the ratio of *PF* (*RPF*). *RPF* was defined as the amount of *PF* after the hemodialysis session, divided by the amount before. *RPF* was calculated separately for 330/380 nm *PF* and 370/460 nm *PF*.

For this analysis, "*change-mode*" was defined as either the difference between two values, or the ratio (value1/value2) or the inverted ratio (value2/value1). Similarly, relative spectral intensities were defined in three so-called "*relativity-modes*" as the difference between intensities in the same spectrum at two wavelengths, the ratio (intensity at wavelength 1 divided by the intensity at wavelength 2) and the inverted ratio (intensity at wavelength 2 divided by the intensity at wavelength 1).

As a first step, the changes before and after dialysis (all three *change-modes*) in spectral intensities per nanometer wavelength and in relative spectral intensities (in all three *relativity-modes*) were tested for correlation to *RPF* using Spearman's ranked correlation tests. A computer program was written to perform these tests for each separate wavelength (per nm) and for all possible wavelength combinations in the entire 350 – 675 nm range.

The results obtained were visualized by plotting the *p*-values of the correlation tests as a function of the wavelength. For the changes in the relative spectral intensities, surface plots were generated where a gray-scale represented the *p*-value at all wavelength pairs. Separate plots were generated for both *PF* wavelength pairs and for all different *change-modes* and all different *relativity-modes*. The plots were visually inspected to find regions of interest (ROIs) where correlations ($p < 0.05$) occurred for several successive wavelengths. figreffig:p-map in the main article provides an example of a surface plot, in which several regions were observed where *p*-values are low for a continuous region. These ROIs were marked by the arrows.

Based on the wavelength ROIs, those wavelength ranges were selected where spectral intensity changes showed the strongest correlation with *RPF*. From these wavelength ranges, so-called spectrum characteristics were defined.

Selection of the *change-mode*

The change of the spectrum characteristics was now defined as the three *change-modes* as described above (difference, ratio or inverted ratio). This resulted in possible correlations with *RPF* but not necessarily linear. Since for the multiple linear regression only linear relations may be used with normally distributed sets, normality and linearity were assessed with Kolmogorov–Smirnov and Pearson's tests respectively. First, for each spectrum characteristic, non-normally distributed sets were excluded. Then, based on the highest Pearson correlation coefficient, one of the three *change-modes* was selected.

CHAPTER 7

General overview and discussion

Cardiovascular diseases are accounting for almost one third of all deaths worldwide.[1] The early detection of an increased cardiovascular risk may be a very helpful tool in prevention and health care. The measurement of autofluorescence (*AF*) of the skin is a non-invasive method for the assessment of the amount of advanced glycation end products (AGEs). These AGEs accumulate in tissues with a low collagen turn-over, such as arteries and skin,[2] and cause e.g. arterial stiffness. Their amount correlates to and is an independent predictor of cardiovascular disease, especially in diabetes mellitus but also in other conditions such as renal disease. This is supported by many studies.[3–14]

Skin *AF* can be measured fast and non-invasively with the AGE Reader. The AGE Reader allows for an assessment of cardiovascular risk by just placing the patient's lower arm on the desktop device. One number, the skin *AF* value, is measured in less than 30 seconds and immediately presented. To partly compensate for variations in absorption and scattering of light by the tissue, skin *AF* is calculated by dividing the average emitted light intensity per nm in the 420 – 600 nm range by the average reflected excitation light intensity per nm in the 300 – 420 nm range, and multiplied by 100. Skin *AF* is expressed in arbitrary units.

This dissertation aimed at resolving research questions, from a biomedical engineering perspective, to improve the measurement and applicability of skin *AF*.

Fluorescence

The AGE Reader utilizes a UV blacklight tube light source that emits light in a rather broad spectrum in the 350 – 405 nm range. Several fluorophores are known that may be excited by this light.[15] This raised the question which of these fluorophores contribute to the discrimination of patients with increased cardiovascular risk and whether they can be measured separately. And more importantly, whether the use of more narrow excitation bands may improve this discrimination. Furthermore, it is unknown to what extent changes in the fluorophore content in the blood vessels influence the values of skin *AF*.

In Chapter 2, the possible advantage of using a more specific excitation peak was studied. In this study, diabetes mellitus (DM) patients with chronic complications, who are known for their high skin *AF* values,[8] were compared with patients without complications, having intermediate skin *AF* values, and with control subjects. Our results showed no improvement of the detection of increased cardiovascular risk if narrower excitation peaks were used. Moreover, no distinct features were observed in

the emission spectra, as obtained with these narrow peaks, that could distinguish the various patient groups. This study suggests that the broad excitation peak is sufficient for the assessment of increased cardiovascular risk.

The study in Chapter 2 showed that the shape of the emission spectra resulting from narrow excitation peaks was similar for all patient groups, although differences in emission spectra did occur for the distinct excitation peaks. We did expect to find some changes between the groups because of the essential role of AGEs in the development and progression of chronic complications in DM.[3, 5, 16] We speculated that the same pool of fluorophores causes fluorescence in all groups, only in a different amount.

The specific excitation and emission maxima of fluorophores should in principle enable the detection of fluorophores from in vivo measurements. Early attempts showed proof of this principle in solutions with negligible scattering.[17] Some authors, such as Durkin et al. and Müller et al. have tried to compensate for absorption and scattering and attempted to quantify specific fluorophores in tissue. The major problems in this respect are still the overlapping nature of the various fluorophores and the inhomogeneous and layered nature of tissue.[18, 19] Bogaards et al. and Stamatas et al. have focused on the quantification of specifically selected fluorescent markers, such as protoporphyrin IX[20] and salicylic acid.[21]

Although the techniques for detection and quantification of specific fluorophores in vivo are not yet readily available, the relative and qualitative change in concentration of certain fluorophores might already be detected. The pathological conditions as often found in patients suffering from DM may give rise to such changes. Therefore, we anticipated that the emission spectra from fluorescence at the narrow excitation peaks might show distinct features unique for the two groups of DM patients (type 1 and type 2) suffering from complications. On the other hand, the overlapping of excitation and emission spectra of the fluorophores involved may obscure this effect. Our results did not show such features. As stated above, we did detect changes in the emission spectra as a function of the excitation wavelength, but these changes were present for all subject categories, even in the healthy control subjects. No specific wavelengths were found that would indicate a relative change in concentration of certain compounds that could enhance the distinction between the various groups. Several complications in DM, such as neuropathy, retinopathy and nephropathy, often occur in a sequence, suggesting that every additional complication that a patient suffers from is an indication of the progression of the disease. This effect may explain the lack of distinct features in the emission spectra of patients from the patient groups

as measured in Chapter 2.

It may be argued that the intensity spectrum of the UV light source in the AGE Reader is rather narrow if compared to the total region in which fluorophores may be excited that are of interest in the assessment of cardiovascular risk. The review of Bachmann et al.[15] listed several possibly relevant fluorophores with excitation maxima ranging from well under 300 nm for tyrosine, tryptophan and elastin, to 450 nm for elastin and collagen crosslinks, FAD and porphyrins. The light source in the AGE Reader emits light in a 50 nm range, centered around approximately 370 nm. Therefore, the AGE Reader may not make use enough of other fluorophores with potentially diagnostic value. The excitation/emission matrix scanner (EEMS) setup may be modified with a different light source in order to explore skin *AF* in the complete 250 – 450 nm range. However, broadening the range has some disadvantages as well. First, lower wavelengths in the UV are increasingly dangerous for human tissue. The legal limitations for intensities used may inhibit the in vivo diagnostic value of a device using these low wavelengths. Second, if a very broad wavelength range is used for illumination, fluorescence crosstalk may occur, where the emission light of some fluorophores may be reused as excitation light for other fluorophores. This greatly complicates the interpretation of a measured emission spectrum. Although measuring skin *AF* with several narrower excitation peaks successively may solve the crosstalk, this also complicates the measuring procedure.

In Chapter 6, it was found that skin *AF* does not change after a hemodialysis session. Hemodialysis partly replaces the renal function, removing several compounds from the plasma, including fluorescing AGEs. This was measured as total plasma fluorescence, also using similar excitation and emission wavelengths as are used for the assessment of skin *AF*. The amount of plasma fluorescence decreased significantly. This suggests that fluorescence from molecules in the blood stream have a very limited or no immediate influence on skin *AF*. Noordzij et al. showed a significant increase of skin AF, up to 10%, two to four hours after an AGE-rich meal.[22] Because they could not attribute this rise to meal-induced glycemic changes, they suggested that skin *AF* was directly influenced by the AGE content of the meal. It has been suggested that the balance of AGE cross-linking and the degradation of AGE cross-linked proteins in the tissue is a dynamic but slow process. The slow removal of AGEs from the tissue by breakdown of its proteins with AGE-cross-links into the bloodstream as AGE free adducts or AGE-peptides during hemodialysis will never be as fast as the penetration of food-derived AGEs from the bloodstream into the tissue. If skin *AF* is not influenced by the AGEs in the bloodstream, as our results

suggested, this may explain the lack of change in skin *AF* in our study in light of the increased skin *AF* in the study of Noordzij et al. In the meantime, although our results showed that skin *AF* for the assessment of cardiovascular risk may be measured either before or after dialysis, it is advisable to be cautious in measuring skin *AF* if a large change in the AGE load is expected, because of e.g. glycemic or oxidative stress or exogenous.

The study in Chapter 6 also showed a significant reduction in 380 nm plasma fluorescence emission upon 330 nm excitation after the dialysis session. However, the reductions of fluorescence at both wavelength pairs (330/380 nm and 370/460 nm) were not equal. Another useful expansion of this study would therefore be to measure complete excitation/emission matrices before and after the dialysis session. This may further increase our knowledge on the reduction of fluorescent compounds in tissue by hemodialysis.

In the current studies, fluorescence is assessed diffusely, by measuring a relatively large part of the skin. An advantage of this approach is that the fluorescence signal is integrated over a larger part of the skin and thereby avoiding local differences. However, in this way, many fluorophores may be excited by the light in the excitation range used, as already stated above. AGEs do not accumulate in the epidermis and therefore, all fluorescence from the epidermis is confounding the AGE assessment. Spatial fluorescence, e.g. by fluorescence tomography, may be used to avoid fluorescence by other fluorophores than AGEs. Furthermore, with fluorescence lifetime imaging (FLIM), the specific fluorescence lifetime of AGEs may be used to distinguish AGEs from other fluorophore that emit light at the same wavelengths. A promising tool that combines these both techniques is the DermaInspect (JenLab, Jena, Germany).[23] In this device, two-photon excitation is used as focusing mechanism to produce tomographs. A focused light beam is used and in a small volume around the focus, a sufficiently high photon density is reached so that two photons may cooperate to excite a fluorophore. The energy needed for excitation originates from both photons together, which may therefore both have only half of the energy needed normally, and thus twice the wavelength. To excite fluorophores in the 350 – 500 nm range, near infrared light in the 700 – 1000 nm range is used. This method allows for excitation in voxels of approximately 1 μm^3. Koelher et al. used the DermaInspect in an in vivo study assessing the aging of the dermis.[24] They could correlate changes in the composition and morphology of the dermal matrix to subject age with $R^2 = 0.9$ in 18 healthy volunteers. These changes may well be caused by AGE cross-linking. Although fluorescence was measured, excitation was at 410 nm (photons of 820 nm),

and no quantitative assessment was performed. With somewhat lower wavelengths, it may be possible to excite the skin AGEs that are assessed with the AGE Reader. However, a current drawback of the technique, besides the considerable cost of the device, is the limited maximum measuring depth of approximately 100 μm,[23] which extends only into the papillary dermis. Therefore, it may not yet be possible to quantitatively assess skin AGE levels with this technique.

Time-resolved fluorescence spectroscopy (TRFS) is an even more general technique that utilizes the fluorescent life-time of fluorophores. Chorvat and Chorvatova reviewed the latest advances in these techniques.[25] Although they briefly mention fluorescence by AGEs, the assessment of AGEs with fluorescence life-time spectroscopy is still a new field. Katika et al. devised an instrument to measure fluorescence life-time in vivo.[26] The light-source used was an LED emitting at 370 nm. Three distinct life-times of fluorescence emission at 442 nm were found. They speculated that the measured life-times of 0.4 and 2.7 ns originated from free and bound NADH respectively and the 9.4 ns life-time from AGEs. However, in a later study from the same group, no increase of this longer life-time fluorescence was found in diabetic subjects.[27] They speculated that the use of the broad excitation wavelength range as used in our studies may explain these differences from the AGE Reader results. Despite these differences, their results indicate that TRFS is a promising technique to distinguish AGEs from other fluorophores.

Reference values of skin *AF*

Skin *AF* may be used as an absolute marker for the assessment of cardiovascular risk. A higher value is simply associated with a higher risk. Nevertheless, in some situations, e.g. in research settings, it is more important to assess the relative risk as compared to other subjects in the same situation. Subject age is the most important risk factor in cardiovascular disease. In Chapter 4, reference values of skin *AF* in a Caucasian healthy population were therefore provided as a function of age. These aid in the detection of increased cardiovascular risk.

It was observed that skin *AF* values increased linearly with subject age, as had been suggested before.[28–30] The accumulation of AGEs is a process involving many reactions,[31] all with their own reaction kinetics. AGE accumulation is a process resulting from the load to which the body is exposed. If this load is constant in time, an exponential decay of the accumulation rate may be expected from these non-enzymatic reactions. On the other hand, also the kinetics of breakdown of AGE-linked

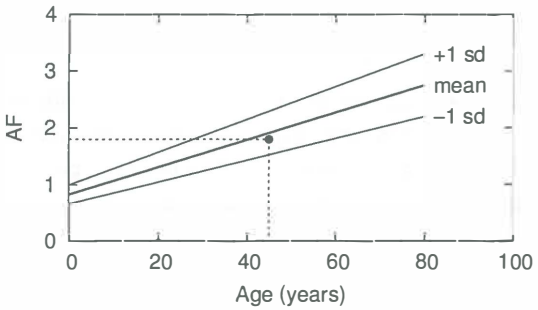

Figure 7.1: *Representation of the measured skin AF value in a graph with reference values. A similar plot is provided in a patient report from the AGE Reader software. The dot represents the skin AF value and calendar age of the subject. The solid lines represent the mean skin AF value for each calendar age and its standard deviation. The reference lines are derived from Koetsier et al.*[32]

proteins and the subsequent excretion of AGE-free adducts, free AGE and AGE peptides should be taken into consideration. This complex nature of AGE accumulation suggests that a maximum amount may exist for the amount of collagen-linked AGEs. Indeed, skin *AF* seldom exceeds the value of 5. Furthermore, higher values of skin *AF* occurs less frequently because subjects with these values have an decreased chance of survival. This may skew the distribution of skin *AF* values, especially in groups of subjects with a higher age. It is therefore remarkable that the trend that was observed is linear and has a proportional standard deviation in a broad age range (0 – 70 years).

In Chapter 4, reference values of skin *AF* were presented in two ways. First, a formula was provided that allowed the calculation of an expected value as a linear function of subject age. Also the expected standard deviation from the mean was found to be a fixed percentage (20%) of the mean. Secondly, a table was presented that provided mean skin *AF* values (and standard deviations thereof) for each age decade. This will provide clinicians with an easy indication of expected skin *AF* for a patient. The current AGE Reader software can show a graph to the user where lines indicate the mean skin *AF* values and one and two standard deviations in both directions for the 0 – 80 years age range. A similar plot is presented in Figure 7.1.

However, these presentation methods may be extended. Well-known alternative clinical presentation methods are calculating a standard score or a percentile. However, it should be kept in mind that skin *AF* itself is related to the risk of cardiovascular

disease or other complications. Therefore, a relative risk may obscure the immediate risk that is reflected by skin *AF* itself.

Another way to use the relation between skin *AF* and subject age is to provide the patient with a so-called AGE-age. The value of skin *AF* can easily be translated into the AGE-age by reversing the linear relation that was provided. Confronting a patient with a higher AGE-age than his own calendar age might raise the awareness of his risk, and may have motivational or didactic reasons. It has even been suggested that this AGE-age is a better estimate of biological age than the calendar age. It is, therefore, also used as a replacement for calendar age in other risk engines such as that of the UK Prospective Diabetes Study.

In Chapter 5, a new algorithm for calculating skin *AF* from measured emission and reflectance spectra was designed for healthy control subjects and tested in a separate group, consisting of subjects of African and Asian descent. The method was based on the deviation of uncorrected skin *AF* from an expected value based on the subject age, derived from Chapter 4. With this method, the reference values as obtained in Caucasian healthy subjects were imposed on subjects of other descents. This might raise the concern about whether people in other ethnic groups have a similar accumulation of AGEs over time. However, even if this is not the case, the advantage of this method is that the reference values of skin *AF* are now linked to healthy subjects, irrespective of ethnic descent. This enhances comparison of populations. Other aspects of the relation between skin color and reflectance, and skin *AF* level are summarized below.

It has been shown that food is an important source of AGEs. The group of Vlassara et al. has published extensive lists of food products and their AGE content.[33, 34] They also reviewed studies in which a higher intake of AGE-rich food is associated with a lower life-expectation. Although the extent to which diets are influencing the accumulation of AGEs in tissue is debated and probably depends on other conditions affecting AGE levels like renal failure, it seems reasonable to assume that eating habits of certain populations may give rise to mean skin *AF* values that are different as compared to the reference values that we have published in Chapter 6. Therefore, caution is advised if two populations are compared. To solve these questions, it is suggested that the AGE Reader is used in future studies assessing the impact of diets and other life-style differences.

Another issue that was raised in Chapter 4 concerned the possible differences between the autofluorescence reader (AFR) and the AGE Reader. In Chapter 1, a brief history of the development of the AGE Reader was provided. The design of

the AGE Reader has been based on the AFR and special care was taken to maintain a similar optical setup and to yield similar skin *AF* values. A small pilot study in 25 subjects with a broad range of skin *AF* values has been performed and showed a good resemblance between values of both devices, as described in more detail in Chapter 3. However, the comparison had not been published. Several skin biopsy validation studies have been performed using the AFR instead of the AGE Reader. Moreover, some validation studies have been performed with the earlier version of the AFR which used a different light source for excitation, with a somewhat lower wavelength maximum. The study for reference values provided a good opportunity to compare the devices and to link the AGE Reader to the validation studies with the AFR.

Reflectance spectra for the adaptation of skin *AF*

A major confounder in measuring skin *AF* with the AGE Reader is the color of the skin. The calculation of skin *AF* as the ratio between the emission and excitation intensities partly compensates this. Still, a dark skin color, i.e. Fitzpatrick skin type V or VI, severely decreased the measured value of skin *AF*. Even in Caucasian subjects, a small, insignificant increase in skin *AF* values was detected for subjects with a lighter skin color. A small pilot study with the AGE Reader in subjects with a dark skin color showed that even in these subjects, skin *AF* is increased in DM patients with chronic complications (non-published clinical data). We therefore hypothesized that a description of the skin color might enable the adaptation of skin *AF* values as a function of skin color.

The AGE Reader instrumentation can also be utilized to measure diffuse reflectance spectra of the skin in the 350 – 675 nm range. This enables the objective description of skin color. Because this measurement is performed at approximately the same time and at the same location of the skin, it provides additional information on the skin under the same conditions as where skin *AF* is measured.

Chapter 5 focused on the improvement of skin *AF* measurements in subjects with darker skin colors. The color of the skin is a result of wavelength dependent scattering and absorption of light in the tissue. Under normal, non-pathological conditions, the amount and distribution of melanin is the major determinant of the color.[35] In our study, several characteristics from the emission and reflectance spectra were found and used to describe the influence of skin color on the *AF* signal. This provided the insight that mainly two characteristics were of influence: melanin index as calculated

in the 360 – 390 nm range and *RedLnSlope* as calculated from the 630 – 675 nm range. In both ranges, the major absorber is melanin.[36] The finding in Chapter 5 that the influence of skin color on skin *AF* can be described for 80% (adjusted R^2) as a function of these two parameters, together with subject age, confirms the major role of melanin absorption in the wavelength range used in the AGE Reader.

The other major absorber in human tissue is hemoglobin. Hemoglobin appears in the skin mostly in two forms: oxygenated and de-oxygenated. These two forms have different absorption spectra and both have strong absorption peaks in the 400 – 600 nm wavelength range,[37] where *AF* emission is measured in the AGE Reader. Therefore, we expected that spectrum characteristics that were related to hemoglobin would have an effect on the measured skin *AF* as well. In a recent study, Noordzij et al. performed ischemia and hyperemia experiments in human volunteers and found differences in skin *AF* for these conditions.[38] Besides the possible role of changes in levels of fluorophores, mainly NADH under these conditions, this effect may be related to changes in hemoglobin absorption. However, in Chapter 5, no relation between hemoglobin related characteristics and the decrease of skin *AF* in subjects with a dark skin color was found. This suggests that the apparent effects of ischemia and hyperemia on the value of skin *AF* are not different as a function of skin color. However, care has to be taken if skin *AF* is measured in patients during ischemia or hyperemia.

In Chapter 5, skin *AF* was measured in patients with no increased cardiovascular risk. The next step would be to measure skin *AF* in DM patients using the new algorithm, that was developed in this study, to find out whether the same increase in skin *AF* values occurs in DM patients irrespective of skin color. A pilot study in a few patients with a dark skin color from the diabetes outpatient clinic of the University Medical Center of Groningen, the Netherlands, was promising. Two larger studies in Hindustan and Creole populations, each including cohorts of DM patients with and without diabetes-related complications and healthy control subjects, have recently been completed and are now analyzed. These studies should confirm the validity of the method to adapt skin *AF* values for skin color.

Reflectance spectra as diagnostic tool

Besides aiding in the adaptation of skin *AF* values, a description of skin color may also provide information about physiological conditions. An obvious example of this effect is erythema: a red skin indicates hyperemia of the capillaries in the lower

layers of the skin and may be a sign of local or systemic inflammation. Another example is the hyper-pigmentation as often seen in patients with kidney failure.[39] Reflectance spectra, that can be measured with the AGE Reader, could ultimately aid in the detection and follow-up and monitoring of several conditions that are related to skin color changes.

In Chapter 6, changes in the skin emission and reflectance spectra of end stage renal disease (ESRD) patients before and after one dialysis treatment session were analyzed. Expressed as so-called spectrum characteristics, several changes occurred. These changes could even be used to describe the decrease of plasma fluorescence from samples taken immediately before and after the dialysis session. In future research, these or other spectrum characteristics may be used to assess effects of interventions or progression of other conditions as well. An example might be to use changes in reflectance characteristics as a marker of dialysis efficiency. Another technique may be to exploit the relation between two or more spectrum characteristics, that may be different in certain patient groups.

Conclusions and future perspective

The present studies provide an improvement and extension of use for the measurement and interpretation of skin *AF*. Skin *AF* values based on the broad wavelength range, as applied in the AGE Reader, adequately discriminate groups of patients with increased cardiovascular risk. Excitation in lower wavelength regions may enable the measurement of more skin fluorophores that could enhance the distinction between several patient groups. The influence of fluorescence of molecules in the blood stream on the value of skin *AF* needs to be explored further. The use of time-resolved fluorescence spectroscopy may improve the distinction between AGEs and other fluorophores.

Baseline reference values of skin *AF* as a function of subject age are now provided and may be used regardless of skin color. Users of the AGE Reader may further explore how the value of skin *AF* is best presented in relation to these baseline values.

Several methods were explored or utilized to yield skin *AF* values that are not dependent on skin color. The careful design of the measuring device minimized the influence of specular reflectance. A theoretical consideration regarding intrinsic absorption had already led to the calculation of skin *AF* as a ratio of the emission light and the reflected light in the excitation range. In earlier versions of the AGE Reader software, these measures were regarded as adequate for subjects with a reflectance in

the UV range of 10% or higher (roughly equivalent to a Fitzpatrick skin photo-type up to IV). An empirical correction was developed to adapt measured skin *AF* values based on characteristics of the reflectance spectrum. This enabled the measurement of skin *AF* values in subjects with almost any skin color. Research is underway that further explores the validation of this method and elucidates whether skin *AF* is also sufficiently reliable to be used to assess cardiovascular risk in subjects with a dark skin color.

Finally, the use of skin reflectance spectra was shown to be valuable in the assessment of hemodialysis effectiveness. The use of this new application of the AGE Reader as a diagnostic tool in various diseases should be explored in the future, possibly in combination with the skin *AF* measurement.

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Summary

The early detection of an increased risk of cardiovascular diseases may be a very helpful tool in prevention and health care. The measurement of autofluorescence (*AF*) of the skin is a non-invasive and very fast method for the assessment of the amount of advanced glycation end products (AGEs). These AGEs accumulate in tissues with a low collagen turn-over, such as skin, and their amount independently predicts an increased cardiovascular risk.

Skin *AF* can be measured with the AGE Reader. To compensate for variations in absorption and scattering of light by the tissue, skin *AF* is calculated by dividing the average emitted light intensity per nm by the average reflected excitation light intensity per nm. This dissertation aimed at resolving research questions, as introduced in Chapter 1, from a biomedical engineering perspective to improve the measurement and clinical applicability of skin *AF*.

In Chapter 2, the possible advantage of using a more specific excitation peak was studied. In this study, measurements on diabetes mellitus (DM) patients with chronic complications were compared with those of patients without complications and control subjects. Our results showed no improvement in discerning these groups if narrower excitation peaks were used. Moreover, no distinct features were observed in the emission spectra, as obtained with these narrow peaks, that could distinguish the various patient groups. This study suggests that the broad excitation peak is sufficient for the assessment of increased cardiovascular risk.

In Chapter 3, skin *AF* values as obtained with the AGE Reader were compared to values as obtained with its predecessor, the AutoFluorescence Reader. The study shows that both devices yield the same values.

In Chapter 4, reference values of skin *AF* in a Caucasian healthy population were provided as a function of age. Subject age is the most important risk factor in cardiovascular disease and skin *AF* may be used as an absolute marker for the assessment of cardiovascular risk. A higher value is associated with a higher risk. Nevertheless, in some situations it is more important to assess the relative risk as compared to other subjects in the same situation, i.e. of the same age. A remarkably linear relation was found between skin *AF* values and subject age. The presented reference values may aid in the improved detection of increased cardiovascular risk.

Chapter 5 focused on the improvement of skin *AF* measurements in subjects with darker skin colors. Under normal, non-pathological conditions, the amount and distribution of melanin is the major determinant of skin color and this substance proved to be the main confounder in measuring skin *AF*. In this study, characteristics from the reflectance spectra were used to describe the influence of skin color on the

AF signal. With this empirical approach, skin *AF* could be adapted such that the measurement of skin *AF* now yields similar values for each age group, regardless of skin color.

In Chapter 6, changes in skin *AF*, skin reflectance spectra and plasma fluorescence were analyzed between before and after a single hemodialysis treatment. It was found that characteristics of the reflectance spectra could be used to describe the decrease of plasma fluorescence in dialysis. Skin *AF* was not different after dialysis, suggesting that skin *AF* for the assessment of cardiovascular risk may be measured either before or after dialysis.

In Chapter 7, the main issues addressed in this dissertation were discussed in a broader perspective and future research suggestions were provided. Excitation in lower or higher wavelength regions may enable the measurement of more skin fluorophores that could enhance the distinction between several patient groups. The influence of fluorescence of molecules in the bloodstream, e.g. due to food intake, on the value of skin *AF* needs to be explored further. Using the improved calculation method of skin *AF*, it should be verified to which extent skin *AF* can also be used to assess cardiovascular risk in subjects with a dark skin color. Finally, the use of skin reflectance spectra as a diagnostic tool in various systemic diseases, possibly in combination with the skin *AF* measurement, should be explored further.

Nederlandse samenvatting

Het meten van autofluorescentie (*AF*) van de huid is een niet-invasieve en zeer snelle methode voor het bepalen van de hoeveelheid hierin aanwezige Advanced Glycation End Products (AGEs). Fluorescentie is een optisch fenomeen waarbij een stof licht weerkaatst na het omgezet te hebben in een andere kleur licht met een hogere golflengte. Het oorspronkelijke licht wordt excitatielicht genoemd, het weerkaatste licht met hogere golflengte heet emissielicht. AGEs betreffen een grote groep moleculen, waarvan sommige fluorescente eigenschappen bezitten. AGEs zijn het metabole eindproduct van een serie reacties waarin suikers, eiwitten en vetten een hoofdrol spelen. Ze stapelen zich op in weefsels met een langzame afbraak van collageen en andere eiwitten, zoals de huid (dermis). Deze stapeling wordt versneld door ziekten zoals suikerziekte (diabetes mellitus) en nierlijden. De hoeveelheid AGEs — en daarmee ook de fluorescentie van de huid — is een onafhankelijke maat in het voorspellen van risico op hart- en vaatziekten en is een belangrijk hulpmiddel in de preventie en behandeling van deze aandoeningen.

Huid *AF* kan gemeten worden met de AGE Reader. Dit is een klein apparaat, ontwikkeld binnen de Rijksuniversiteit Groningen en het spin-off bedrijf DiagnOptics Technologies, dat de meting van huid *AF* op een patiëntvriendelijke manier aan de onderarm mogelijk maakt. Om te compenseren voor variaties in absorptie en verstrooiing van het licht door de huid, wordt huid *AF* berekend door de gemiddelde intensiteit van het gemeten emissielicht per nanometer (nm) te delen door de gemiddelde intensiteit van het gereflecteerde excitatielicht per nm. Hiermee geeft de AGE Reader één getal dat de hoeveelheid AGEs in de huid weergeeft. In dit proefschrift zijn de onderzoeksvragen, zoals geïntroduceerd in Hoofdstuk 1, benaderd vanuit een biomedisch technologisch perspectief, teneinde de meting en klinische toepasbaarheid van huid *AF* te verbeteren.

In Hoofdstuk 2 werd het mogelijke voordeel van het gebruik van meer specifieke excitatiegolflengten bestudeerd. In deze studie werden *AF*-waarden gemeten met veel smallere excitatiepieken dan die in de AGE Reader gebruikt worden. *AF*-waarden van diabetes mellituspatiënten met chronische complicaties, zoals hart- en vaatziekten, retinopathie en neuropathie, werden vergeleken met de waarden van patiënten zonder deze complicaties en van een controlegroep van gezonde proefpersonen. Het gebruik van de smallere excitatiepieken leidde er niet toe dat deze groepen beter van elkaar konden worden onderscheiden. Bovendien werden er geen specifieke kenmerken waargenomen in de emissiespectra, verkregen met deze smallere pieken, die kunnen bijdragen aan het onderscheiden van de diverse groepen. Geconcludeerd werd dat het gebruik van smallere excitatiepieken geen verbetering oplevert voor het vaststellen

van toegenomen risico op hart- en vaatziekten.

In Hoofdstuk 3 werd de AGE Reader vergeleken met zijn voorganger, de Auto-Fluorescentie Reader (AFR). In het verleden zijn diverse studies uitgevoerd met deze AFR die een verband aantonen tussen *AF*-waarden en onder meer het risico op hart- en vaatziekten. De studie liet zien dat beide apparaten vergelijkbare waarden opleveren, waardoor eerdere studies die met de AFR uitgevoerd werden, kunnen worden gebruikt voor de validatie van de AGE reader.

In Hoofdstuk 4 werden leeftijdsafhankelijke referentiewaarden van huid *AF* in een populatie van gezonde, blanke proefpersonen vastgesteld. Er werd een opmerkelijk lineaire relatie tussen huid *AF*-waarden en leeftijd gevonden. Hoe ouder een persoon is, hoe meer kans hij heeft om hart- en vaatziekten te ontwikkelen, en dus is de leeftijd van een persoon de belangrijkste risicofactor in hart- en vaatziekten. De *AF*-waarde van de huid kan gebruikt worden als een absolute maat voor het bepalen van dit risico. Een hogere waarde is gerelateerd aan een hoger risico. Desondanks is het in bepaalde situaties interessanter om het relatieve risico in vergelijking met patiënten in dezelfde situatie in te schatten, bijvoorbeeld ten opzichte van mensen van dezelfde leeftijd. Hierdoor is het mogelijk om andere risicofactoren te onderzoeken. Deze studie liet onder meer zien dat roken een leeftijdsonafhankelijke risicofactor is voor hart- en vaatziekten. De gepresenteerde referentiewaarden dragen bij aan een betere detectie van toegenomen risico op hart- en vaatziekten.

Hoofdstuk 5 beschrijft hoe metingen van huid *AF* bij mensen met een niet-blanke huidskleur verbeterd kunnen worden. Onder normale omstandigheden is de hoeveelheid en verdeling van melanine het meest bepalend voor de huidskleur. Melanine is een kleurstof die wordt aangemaakt in de bovenste laag van de huid, de epidermis, en is een belangrijke beschermer tegen ultraviolet licht. Deze stof bleek de belangrijkste verstorende factor in de meting van huid *AF* te zijn. In deze studie werden karakteristieken van reflectiespectra van de huid gebruikt, bijvoorbeeld de hoeveelheid reflectie bij een bepaalde golflengte ten opzicht van de reflectie in een ander deel van het spectrum, om de invloed van huidskleur op het *AF*-signaal te berekenen. De reflectie is in dit verband gedefinieerd als het percentage licht, per nm golflengte, dat de huid weerkaatst. Dit kan met de AGE Reader (met aangepaste software) gemeten worden, maar staat los van de *AF*-meting. Met deze empirische benadering was het mogelijk de berekening van huid *AF* zodanig aan te passen dat de meting van huid *AF* nu vergelijkbare waarden oplevert voor mensen in dezelfde leeftijdsgroep, onafhankelijk van huidskleur.

In Hoofdstuk 6 werden de veranderingen in huid *AF*, huid reflectiespectra en

plasmafluorescentie tussen metingen voor en na één hemodialyse behandeling geanalyseerd. Plasmafluorescentie werd in vitro bepaald van plasmamonsters die op dezelfde momenten dat de *AF*- en reflectiemetingen werden gedaan, werden afgenomen. De *AF*-waarde van huid bleef na dialyse hetzelfde als de waarde voor dialyse. Voor het bepalen van het risico op hart- en vaatziekten mag huid *AF* dan ook zowel voor als na dialyse gemeten worden. Wel bleek dat de karakteristieken van de huid reflectiespectra de afname in plasmafluorescentie tijdens dialyse gedeeltelijk kunnen beschrijven. Dit suggereert dat deze spectra gebruikt kunnen worden voor het monitoren van diverse (patho-)fysiologische veranderingen die bijvoorbeeld tijdens dialyse optreden.

In Hoofdstuk 7, dat een overzicht geeft van de belangrijkste uitkomsten die in deze dissertatie aan de orde kwamen, werden de resultaten in een breder perspectief bediscussieerd. Verder werden hierin ook suggesties voor toekomstig onderzoek gedaan. Door excitatie in lagere of hogere golflengtegebieden te gebruiken, kunnen mogelijk andere fluorescerende stoffen in de huid gemeten worden die het onderscheid tussen diverse patiëntengroepen kunnen verduidelijken. Tot nog toe heeft onderzoek weinig invloed laten zien van fluorescentie van moleculen in de bloedsomloop, bijvoorbeeld door voedselinname, op huid *AF*-waarden. Dit zou nog nader onderzocht kunnen worden. Gebruik makende van de verbeterde rekenmethode voor huid *AF* zou het bepaald moeten worden in welke mate huid *AF* ook gebruikt kan worden in het bepalen van risico op hart- en vaatziekten in personen met een donkere huidskleur. Tenslotte zou het gebruik van reflectiespectra van de huid als hulpmiddel voor monitoring van metabole veranderingen in diverse stofwisselingsziekten, mogelijk in combinatie met de meting van huid *AF*, verder onderzocht moeten worden.

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