Skin autofluorescence in diabetes mellitus

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Skin autofluorescence in diabetes mellitus

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CHAPTER 1

General introduction and aims of the thesis

Autofluorescence

Tissue can exhibit fluorescence when excited by a light source of a suitable wavelength. This fluorescence emission, arising from endogenous fluorophores, is an intrinsic property of the tissue and is called autofluorescence to be distinguished from fluorescent signals obtained by adding exogenous markers (like sodium fluorescein). The tissue fluorophores absorb certain wavelengths of light (excitation light), transform the energy, and release it again in light of longer wavelengths (emission). Several tissue fluorophores have been identified, such as collagen, elastin, lipofuscin, NADH, porphyrins and tryptophan [1,2]. Each fluorophore has its characteristic excitation and emission wavelength, that enables localization and further quantification of a particular fluorophore. Autofluorescence can be induced in several tissues and can therefore be applied in investigation of several diseases. It is also used to distinguish malignant from benign tissue in several tissues, such as the skin and cervix [3-5]. Furthermore, in ophthalmology, autofluorescence of the lens has been studied in the past in extension [6-8]. It was found that autofluorescence of the lens was increased in ageing and diabetes. Autofluorescence of the lens appeared to be caused by glycation and, subsequent oxidation of lens crystalline. Products of this non-enzymatic glycation and oxidation (Maillard reaction) are also called advanced glycation endproducts (AGEs). Accumulation of AGEs in the lens play a major role in cataract formation in ageing and diabetes.

Increased autofluorescence of the skin in patients with diabetes was a finding by serendipity in 1996. During measurements on capillary sodium fluorescein leakage in patients with diabetes mellitus using a fluorescence microscope setup, unexpectedly high levels of skin autofluorescence were found in patients with diabetes mellitus compared to healthy volunteers [9,10]. This observation was recognized as a potential non-invasive measure for tissue accumulation of advanced glycation endproducts (AGEs). Since the publications about lens autofluorescence and the first studies about tissue AGEs measurements using ‘Collagen Linked Fluorescence’ (CLF) were available [11-13]. AGEs linked to collagen have a characteristic fluorescence spectrum at 440 nm upon excitation at 370 nm and is called CLF. Several publications in the

1980s and 1990s reported accumulation of AGEs in skin or other tissue and its relation to chronic complications of diabetes mellitus [14-17]. In that time, the most frequently studied fluorescent AGE was pentosidine (excitation 335 nm, emission 385 nm), which consists of a crosslink between lysine and arginine residues. Not all AGEs exhibit autofluorescent properties. Examples of non-fluorescent AGEs are N^{ε}-carboxy*methyl*lysine (CML), N^{ε}-carboxy*ethyl*lysine (CEL), and pyrraline. The different pathways of AGE formation and the relation between AGE-accumulation and complications of diabetes and other conditions, like atherosclerosis, renal failure and rheumatic diseases are described in extension in Chapter 3.

Tissue AGE assessments like quantification in extracts from homogenates of skin biopsies using high performance liquid chromatography (HPLC) or CLF, require invasive sampling. Many biochemical and immunochemical assays for measuring fluorescent en non-fluorescent AGEs in serum have been developed over the last decades [18-22]. Each technique has however its own limitation, for example poor relation with tissue contents of AGEs, low reproducibility, or time-consuming and expensive. The advantages and disadvantages of the available AGE-assessment methods are described in detail in a single paragraph of Chapter 3.

The analogy with fluorescence of skin biopsies in previous studies was used to develop a tool aiming to non-invasively assess AGE accumulation: the Autofluorescence Reader (AFR). Skin autofluorescence, non-invasively assessed using the AFR, can be more conveniently applied to large groups of patients and is also more suitable for repeated measurement. Because skin autofluorescence is measured in vivo in the tissue compartment, it might achieve a better representation of tissue AGE-content compared to serum AGE assays.

The assumption that skin autofluorescence was a measure for tissue AGE-content was studied in a validation study previously [23]. Skin autofluorescence was measured in 46 patients with diabetes and in 46 control subjects. Simultaneously, skin biopsies were taken from the lower arm and analysed for collagen-linked fluorescence (CLF) and specific AGEs: pentosidine, CML, CEL (JW Baynes, Columbia USA). This validation study of the AFR showed good relations between skin autofluorescence and fluorescent

and non-fluorescent AGEs assessed in skin-biopsies, with good reproducibility [23]. A later study in the same study group showed that skin AF appeared to be a predictor for cardiovascular complications in diabetes patients [24].

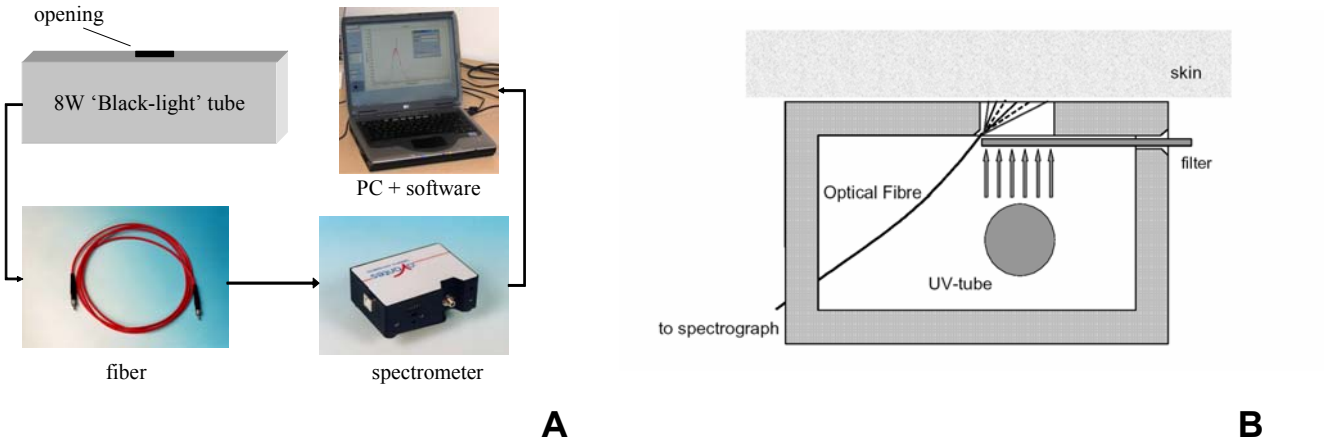

Figure 1. Components of the Autofluorescence Reader (AFR), panel A. Schematic drawing of the light collection section of the AFR, panel B.

The Autofluorescence Reader

The setup of the AFR consists of a light source, a glass fiber that is connected to a spectrometer, and a computer containing the analysing software to which the spectrometer is connected, *Figure 1A*. A schematic drawing of the light collection part of the instrument is given in *Figure 1B*. An 8-Watt blacklight (Philips) was used with the excitation range of 350-420 nm (maximum intensity at 370 nm). The AFR illuminates a skin surface of ~4cm^2, guarded against surrounding light. Emission light and reflected excitation light from the skin are measured with a spectrometer (AVS-USB2000, Avantes Inc. Eerbeek, The Netherlands) in the 300-600 nm range, using 50-µm glass fiber (Farnell, Leeds, UK). The fiber is not in contact with the skin, but is located at some distance from the skin, leading to an integration area of approximately 0.4 cm². This is preferred while the area of the tissue that is seen by the detecting optical fiber, should be large enough to prevent gross variations of the result by particular contributions that occur on small displacements of the probe, such as capillaries and other small blood vessels, hair follicles, sebaceous glands, etc. AFR

measurements were performed at room temperature in a seated position at the volar side of the arm approximately 10cm below the elbow fold.

Reproducibility was tested previously by performing repeated AF measurements taken over a single day in 25 control subjects and diabetic patients and showed a mean relative error in AF of 5.0% [25]. Intra-individual seasonal variance among control subjects and diabetic patients also showed a mean relative error of ~5.0%.
Since skin pigmentation may influence autofluorescence (AF) by light absorption, AF was calculated by dividing the average emitted light intensity per nm in the range 420-600nm (I_{em}) by the average excitated light intensity per nm in the range 300-420nm (I_{exc}), and multiplied by hundred. AF was expressed in arbitrary units (a.u.) [26].

$$AF = I_{em} / I_{exc} \times 100$$

Skin reflection was calculated in the range 300-420nm by dividing the mean intensity reflected from the skin by the mean intensity reflected from a white Teflon block (assuming 100% reflectance). Skin reflection was used in the studies of this thesis to exclude patients that were to dark-skinned, leaving too little light to reliably measure autofluorescence with the current set-up. Measurements were discarded if skin reflection was below <7.5%.

Aims of this thesis

The first aim of the thesis was to evaluate whether the excitation spectrum of the AFR needed to be further optimized to detect possible differences in fluorescence characteristics, as the excitation range of the current used AFR is rather broad and seems therefore less specific. Therefore, we studied whether specific excitation wavelengths could induce different fluorescence peaks corresponding with different fluorophores or AGEs (Chapter 2). We hypothesized a possible difference in biochemical composition of AGEs between type 1 and type 2 diabetes, as these diseases with a different metabolic condition could generate different AGEs. A second

question was whether diabetes patients with complications have different AGEs that result to complications, or that it is just the quantity of AGEs that results in development of diabetes complications. We therefore selected age-matched type 1 and type 2 diabetes patients, both with and without complications, as well as a nondiabetic control group.

The second main focus of this thesis is to further clinically validate the AFR. For this purpose, skin autofluorescence measurements were performed in a large type 2 diabetes population (~1000). The relation of skin autofluorescence to clinically used variables in diabetes care was evaluated (Chapter 4). Furthermore, it was prospectively studied in this cohort, whether skin autofluorescence is a risk indicator for chronic diabetes complications and cardiovascular morbidity and mortality (Chapter 5 and 7).
For this main aim of the thesis, a large population-based cohort of type 2 diabetes patients was needed allowing to discriminate possibly confounding factors and to extrapolate results to clinical practice. In the Zwolle region there was an ongoing epidemiological study in type 2 diabetes patients (ZODIAC-study) [27,28]. Fortunately, we had the opportunity to collaborate with this study group. The Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) study started in 1999 and was embedded in a shared-care project in a primary care population-based cohort of type 2 diabetes patients. These type 2 diabetes mellitus patients are well characterised as they participate in the shared-care project with annual regular standardised assessment of glycemic and metabolic status and chronic diabetes complications. *Figure 2* illustrates the origin of the cohort that had an autofluorescence measurement in 2001, and was included in the follow-up study.
Chapter 6 involves a study where skin autofluorescence was not studied, but where life-expectancy of the ZODIAC-derived type 2 diabetes cohort was specifically studied.

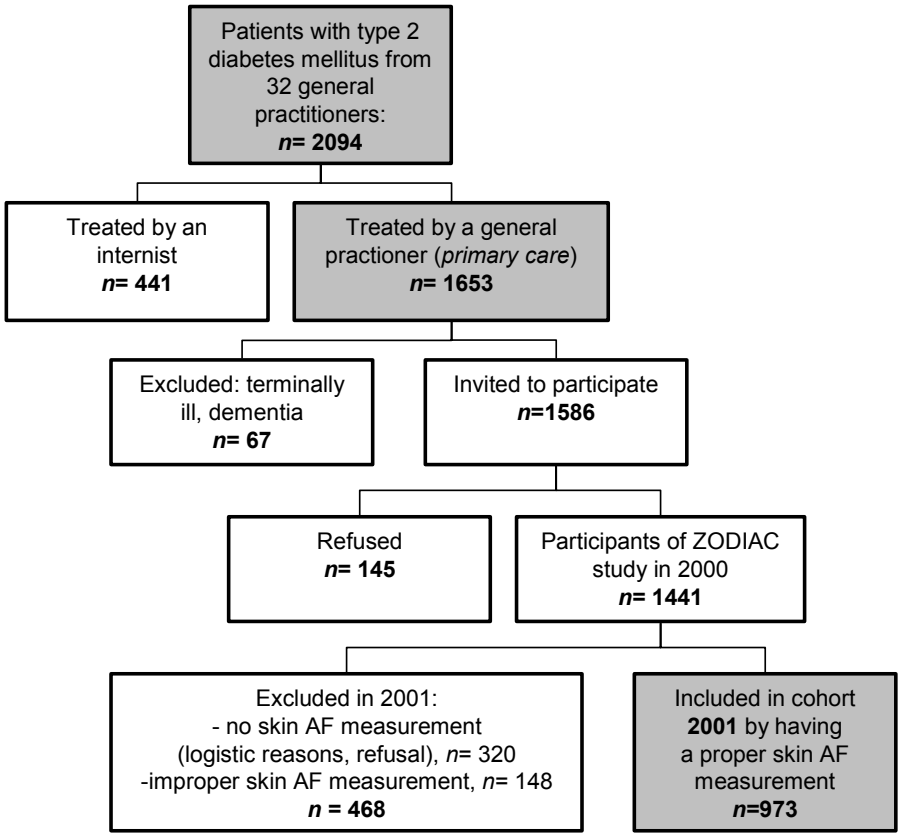

Figure 2. Flowchart of the enrolment of the type 2 diabetes study cohort from 32 general practitioners of a district in the ZODIAC study and further derivement of the type 2 diabetes cohort entering the skin autofluorescence follow-up study.

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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

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

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

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

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

INTRODUCTION

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

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

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

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

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

The aim of this study is first to determine which excitation and emission wavelengths are optimal for differentiation between diabetic and non-diabetic subjects or between diabetic subjects with and without DM-related chronic complications. And secondly, to evaluate whether the presence of DM-related chronic complications is associated with differences in fluorescence spectra.

METHODS

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

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

Table 1: Characteristics of the five groups.

	DM type 1 without compl.	DM type 1 with compl.	DM type 2 without compl.	DM type 2 with compl.	Control
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 ±
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 under an angle of 45 degrees. This glass fiber is connected to a spectrometer (USB2000, Avantes, Eerbeek, The Netherlands). The total setup, referred to as Excitation Emission Matrix Scanner (EEMS) has a similar setup as the previously described tool for measuring skin autofluorescence, the AFR [25]. Wavelength scanning of the monochromator was realized with a PC using a LabView program (National Instruments, Austin, USA). The spectra were obtained by this software as well. Measurements were performed on the volar side of the arm, approximately 10 cm below the elbow. A whole series of measurements, including measurements at 11 excitation wavelengths (355 - 405 nm, in steps of 5 nm) was performed in a few minutes. Optimal integration times were determined by the LabView program, therefore, all spectra were measured in the same dynamic range of the spectrometer. Dark spectra were also obtained and subtracted for all applied integration times. The shape of the peak at each selected excitation wavelength can be described by a normal distribution with a standard deviation of 6.5.

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

$$AF_E(\lambda_{exc}) = 100 \times \langle I_{em} \rangle / \langle I_{exc} \rangle \qquad \textit{equation (1)}$$

where $\langle I_{em} \rangle$ is the mean measured light intensity in the range 420 - 600 nm and $\langle I_{exc} \rangle$ is the mean intensity in the range 300 - 420 nm, both for excitation wavelength λ_{exc}. This same method is used in the AGE Reader to calculate AF from the spectrum. After calculating AFE for all subjects, the mean AFE for subjects in the control group per excitation wavelength was used as a reference for all four DM subject groups.

For comparison between AF values of DM subject groups and the control group, we introduced relative autofluorescence as

$$AF_{E,rel}(\lambda_{exc}) = \langle AF_E(\lambda_{exc}) \rangle_{patient} / \langle AF_E(\lambda_{exc}) \rangle_{control} \qquad \textit{equation (2)}$$

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

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

$$AF_{BL} = \Sigma\, w_i\, AF_i \,/\, \Sigma\, w_i \qquad \textit{equation (3)}$$

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

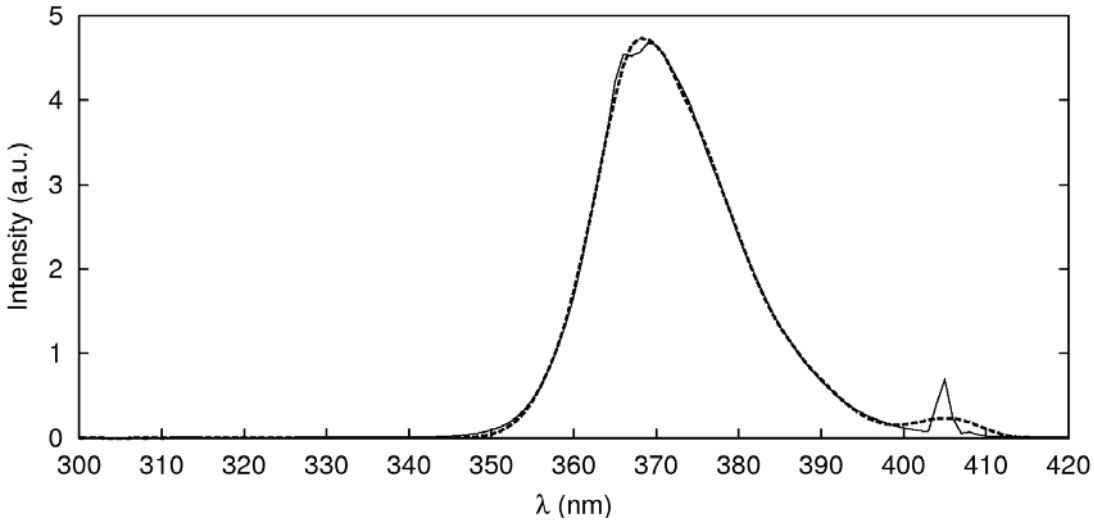

Figure 1. Spectrum of the UV-A light source as used in the AFR and the AGE Reader (continuous line) and a fit using a weighted summation of EEMS responses of separate excitation peaks (dotted line). The small peaks at 366 nm and at 405 nm are caused by mercury emission within the lamp.

To analyze possible differences in the emission spectra between the groups, the spectra were all normalized. The mean value of intensity of the emission peak (in the range 450 - 600 nm) was taken as a reference. Subsequently, mean values were calculated (per nanometer) from the normalized spectra of all subjects in each group for each excitation wavelength. These mean spectra were used to observe whether emission peaks occurred that might be specific for a certain patient group.

RESULTS

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

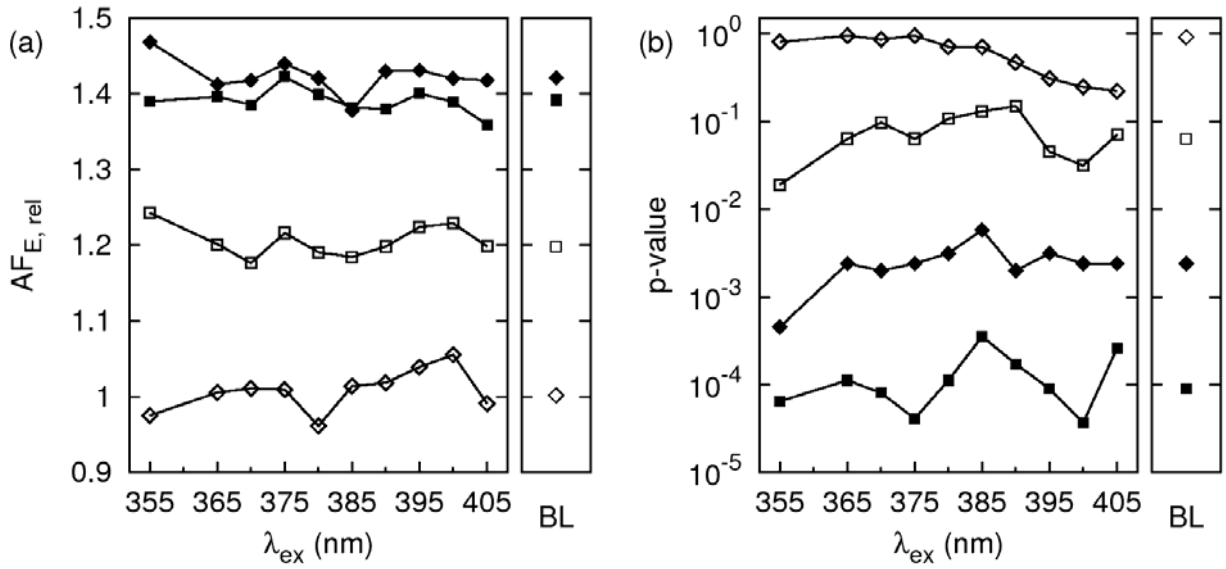

Figure 2. A. Mean relative autofluorescence for the separate excitation peaks ($AF_{E,rel}(\lambda_{ex})$) and the broad excitation peak from the blacklight (BL) as used in the AGE Reader. **B.** Statistical significance (Mann-Whitney U-test) of the differences between AF_E values of subjects in the respective subject groups and the control group. Squares denote the groups of subjects with type 1 DM, diamonds denote type 2 DM. Closed figures denote the groups of subjects with chronic complications, open figures denote the groups of subjects without complications.

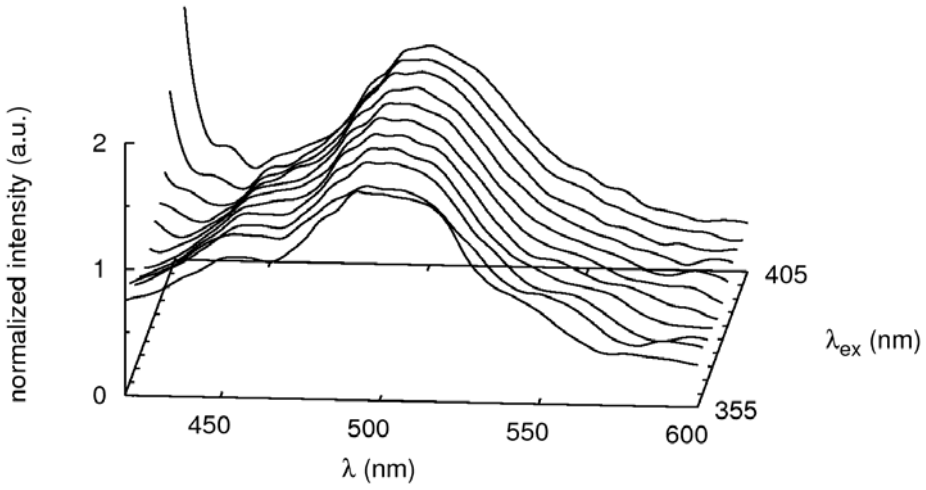

Figure 3. Emission peaks from the average spectra of the control group for increasing excitation wavelength. The spectra are normalized for emission.

Normalized spectra. The emission peaks of the normalized spectra for the control group are displayed in *Figure 3*. The shape of the spectrum is slightly changing for different excitation wavelengths. However, the maximum intensity remains at approximately 500 nm, independent of excitation wavelength. The normalized spectra

of the four patient groups have a similar shape. The emission spectra of all five groups are shown in *Figure 4* for excitation wavelengths of 375 nm (a) and 400 nm (b). These figures clearly show the similarity of the emission spectra between groups. The examples are typical for all other excitation wavelengths as well.

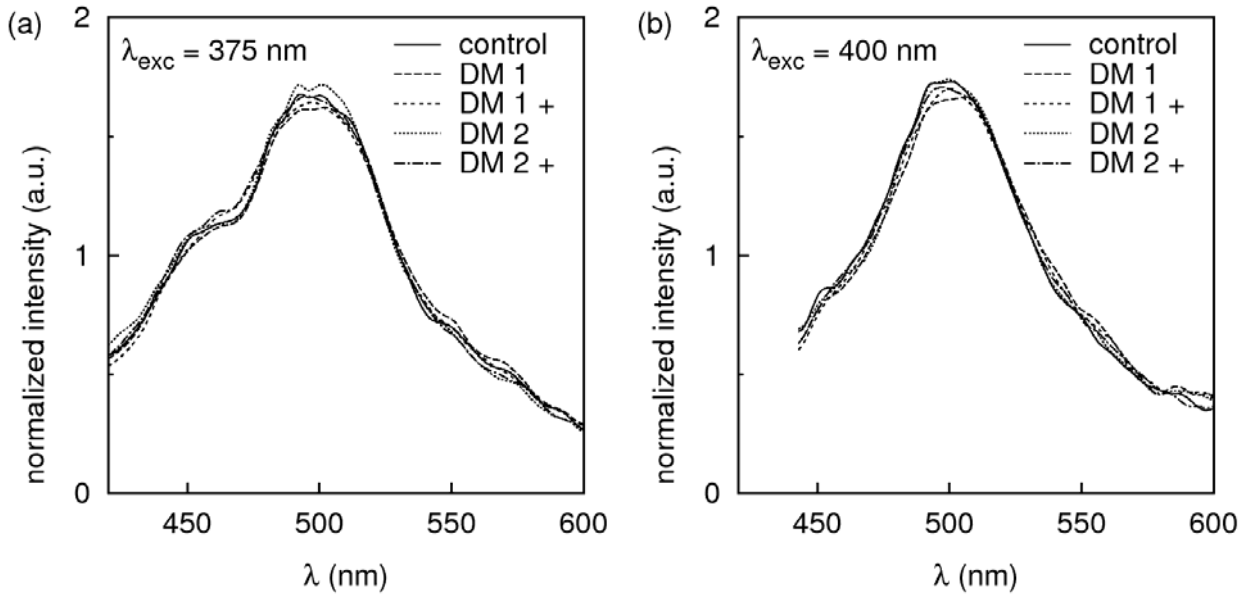

Figure 4. Emission peaks from the average spectra of the five groups for λ_{exc} = 375 nm (a) and λ_{exc} = 400 nm (b). In (b), the spectrum starts at 443 nm as below that wavelength, the excitation peak is still present.

DISCUSSION

No specific excitation or emission wavelengths were found that would yield an increased distinction between the groups of patients with both types of DM, with or without chronic complications, or the control group. In fact, our results show almost constant factors and significances for all excitation wavelengths. Consequently, about the same factors occur for the broad excitation band from the blacklight as used in the AGE Reader. From the observation that the emission spectra of the five groups are very similar for each separate excitation wavelength, it can be concluded that no fluorophores dictate the use of specific excitation or emission wavelengths in these

patient groups. Therefore, the shapes of the spectra do not add more information to skin autofluorescence as determined by the AGE Reader for recognizing DM or chronic complications in DM. Moreover, from our results, all fluorophores seem to be equally increased in the groups of patients with complications.

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

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

Our initial expectation was that we might see specific AGE-related emission peaks in metabolic diseases like DM as compared to control subjects, since AGEs have an essential role in the development of chronic complications in DM [13,38,39]. We furthermore expected a possible difference between type 1 and type 2 DM, as these diseases with different metabolic conditions could generate different AGEs. Collagen-linked 370/440 nm fluorescence, as a marker of AGE accumulation, was a proven predictor of these complications in earlier studies on skin biopsies, and fluorescent AGEs such as pentosidine and argpyrimidine are also related to such conditions. These and some other fluorescent compounds (for example NADH) might result in clinically

relevant specificities in excitation-emission pairs. However, attempts to derive specific emission peaks from our measurements between different groups have not yet been successful; the clinical differences manifest themselves proportionally for all excitation-emission pairs.

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

CONCLUSION

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

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CHAPTER 3

The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation

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ABSTRACT

Advanced glycation endproducts (AGE) are a class of compounds resulting from glycation and oxidation of proteins, lipids or nucleic acids. Glycation is the non-enzymatic addition or insertion of saccharide derivatives to these molecules. This leads to the formation of intermediary Schiff bases and Amadori products and finally to irreversible AGE. This classical view has been modified in recent years with recognition of the importance of oxidative and carbonyl stress in endogenous AGE formation. AGE may also have exogenous sources, in certain foods and tobacco smoke. A whole class of specific and non-specific receptors binding AGE has been characterized. Apart from cross-linking of proteins by AGE, the effects of receptor stimulation contribute to the development of chronic complications of conditions like diabetes mellitus, renal failure, and atherosclerosis. Possible interventions to reduce the effects of AGE accumulation include AGE formation inhibitors or breakers, or receptor blockers, but possibly also dietary interventions.

Some of the problems with current assay or diagnostic techniques, and several unresolved issues on the role of AGE in disease will be discussed. Our review will focus on the clinical and pharmaceutical implications of these developments.

INTRODUCTION

Advanced glycation endproducts (AGE) are a diverse class of compounds resulting from a glycation process under the strong influence of oxidative or carbonyl stress. Although AGE were initially considered as a noxious byproduct with an adverse effect on the function of proteins due to increased cross-linking, it has become evident that AGE also bind and interact with several receptors, such as the receptor for advanced glycation endproducts (RAGE) and also scavenger receptors like galectin-3 and CD36. Binding of AGE to such receptors mediates intracellular signalling and induces changes in cellular function, resulting in release of cytokines and growth factors. The AGE-receptor interactions are now considered pivotal in the AGE-induced pathogenetic changes.

AGE accumulate with age on long-lived proteins, but at a more rapid rate in conditions like diabetes mellitus and renal failure. Diabetes and renal failure may serve as examples of increased AGE formation and breakdown, respectively, but in both conditions the additional role of increased oxidative stress is evident. This review will address in more detail the relation between AGE accumulation and chronic complications of these and related conditions. We also review the developments of the different strategies in reducing AGE accumulation. AGE formation inhibitors (aminoguanidine, pyridoxamine and others), AGE breakers (ALT-711) and other approaches to reduce AGE accumulation will be discussed.

SOURCES OF AGE FORMATION IN THE BODY

Endogenous AGE Formation

It was long believed that glucose, the type of sugar most abundantly contained in an organism, was biologically inert. However, proteins, lipids and nucleic acids can be modified non-enzymatically by glucose and other reducing sugars [1]. In the early stages of AGE formation, the so-called Maillard reaction, the aldehyde groups of reducing sugars react with amino groups of N-terminal amino acids or epsilon lysine to form Schiff bases, which are then converted by the Amadori process into a more stable ketoamine (Amadori product). In most proteins the source of the amino group is the epsilonamino group of the amino acid lysine. Reducing sugars in solution exist in both open-chain and ring (pyranose or furanose) forms. Only the open-chain structures react with proteins. The proportion of the open-chain forms thus determines the reactivity of the sugars in the Maillard reaction with a rank order of glucose < fructose < ribose, with phosphorylated sugars such as intracellular glucose-6-phosphate, being more potent than their unphosphorylated counterparts [2,3] as well. The reactivity of intracellular, phosphorylated sugars is illustrated by a 13.8 fold increase in intracellular AGE content after 1 week of incubation of endothelial cells in the presence of high glucose [4]. Because the glycation process is concentration-dependent in the early rather than later stages of the Maillard reaction, it is enhanced in hyperglycaemia [5,6]. During AGE formation, reactive intermediate products are formed [7,8]. These reactive

intermediate products are also termed in the literature as: dicarbonyls, oxoaldehydes, reactive carbonyl compound (RCO) or ‚carbonyl stress, [9]. Well-known examples are methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) [10]. MGO can be formed from oxidative decomposition of fatty acids, but non-oxidative mechanisms (anaerobic glycolysis) predominate in its formation. Mitochondrial overproduction of reactive oxygen species, with consequent inhibition of the glycolytic enzyme GAPDH and increased triose phosphate levels have a central role in the formation of intracellular MGO-derived AGE [11,12]. Overproduction of superoxide by the electrontransport chain in mitochondria seems not only to be responsible for increased intracellular AGE formation, but also acts as a common pathogenetic pathway for other mechanisms of hyperglycaemia-induced damage like PKC activation, and polyol and hexosamine pathway induction.

The enzyme glycoxalase may have an impairing role in AGE formation by promoting the detoxification of dicarbonyls, as was recently described by Miyata [13]. AGE formation can also be influenced by reducing compounds such as ascorbate. In vitro studies show that anti-oxidants reduce CML (Nε-carboxymethyllysine) formation. Oxidation of polyunsaturated fatty acids in the presence of proteins can lead to CML formation suggesting that lipid oxidation has a role in AGE formation [14]. In inflammatory conditions like rheumatoid arthritis, increased AGE formation arises without hyperglycaemia. An in vitro study showed that myeloperoxidase produced by activated phagocytes, is able to form reactive carbonyls and CML [15]. Thus, besides reducing sugars and peroxidation of lipids, myeloperoxidase seems to play an additional role in the formation of AGE precursors and AGE. This could contribute to the insights of the pathogenesis of various inflammatory diseases.

As a final point in this paragraph of endogenous AGE formation, genetic factors might also contribute to the course of glycation. In a classical twin study, it was found that the interclass correlations in the clinically well-known Amadori product glycated haemoglobin (HbA1c) were considerably higher in monozygotic twins compared with dizygotic twins, independent of an also demonstrable genetic effect on fasting glucose levels [16]. Another mono- and dizygotic non-diabetic twin study

found that, after adjusting for age, current glucose homeostasis and smoking, 28% of the interindividual variation in lens protein autofluorescence was attributable to hereditary factors (and shared environment for 58%, and non-shared environment for 14% [17]. Thus, there are indications that glycation levels are partly genetically determined, but this area has not been extensively studied so far.

Exogenous Sources of AGE: Food and Smoking

The Maillard reaction leading to AGE formation was originally described for heating food. AGE ingested with food are absorbed to a small extent, although the diversity of AGE and assay problems quantification of AGE absorption from food is difficult. Oral bioavailability is estimated to be in the order of 10%, the AGE-induced cross-link formation is resistant to enzymatic or chemical hydrolysis [18]. The accumulation of food derived AGE and alpha-oxoaldehyde AGE precursors has been assumed to contribute to AGE derived deleterious effects. When AGE-modified albumin was administered to healthy nondiabetic rats and rabbits [19], after 2-4 weeks animals displayed diabetes-like vascular complications: a significant increase in vascular permeability, significant mononuclear cell migration in subendothelial and periarteriolar spaces and a defective endothelium-dependent and -independent vasodilatation. Furthermore, glomerulosclerosis and albuminuria, comparable to diabetic nephropathy are seen in control rats receiving daily injection with AGE [20]. Both animal studies showed the deleterious effect of exogenous AGE. An additional human study showed that the daily influx of dietary AGE includes glycotoxins that may constitute an added chronic risk for renal-vascular injury in DM [18]. Dietary restriction of AGE food is discussed below under the Interventions paragraph.

Apart from the direct intake of AGE with food, meals can also influence AGE accumulation and production by excursions in glucose levels. After a meal, diabetic patients can exhibit a hyperglycaemic state, a so-called post prandial glucose excursion (PPGE). These PPGE can even occur when blood glucose is tightly regulated. A study showed that the so-called PPGE correlated well with MGO and 3-DG, two highly reactive precursors of AGE formation [21].

Tobacco smoke contains products that produced AGE-like fluorescence, protein crosslinks, mutagenicity and increased AGE on plasma proteins [22]. Cigarette smoke contains high concentrations of GO and MGO from the thermal decomposition of saccharides. These are the likely important mediators of smoking associated AGE formation. Increased serum AGE levels were found in diabetic smokers compared to diabetic non-smokers [23].

REMOVAL OF AGE

The removal of proteins cross-linked with AGE depends on both the degradation of AGE linked proteins to AGE-peptides by macrophages, and on the clearance of AGE and AGE-peptides by the kidney [24-27]. Macrophages with special AGE receptors engulf and subsequently transport AGE. Miyata et al. provides evidence for filtration of the AGE-compound pentosidine through the glomeruli and the active reabsorption of pentosidine in the proximal tubules [26]. After modification or degradation in the proximal tubules pentosidine was finally cleared in the urine. These findings were confirmed for AGE-peptides by Gugliucci et al. [27]. A fall in renal glomerular or tubular function thus affects the capacity for AGE removal and result in higher blood level of AGE and eventually tissue AGE accumulation.

Scavenger receptors in the liver Kupffer and endothelial cells may also result in endocytic uptake of AGE proteins from the blood [28,29]. High serum AGE levels have been reported in patients with liver failure [30]. The expression of the macrophage scavenger receptor (MSR) which is involved in the endocytic AGE uptake in liver endothelial cells, but perhaps also subsequent steps like lysosomal degradation, may be increased by insulin [29]. It was recently reported that plasma AGE levels are markedly elevated in liver cirrhosis and correlated with the severity of disease, while serum CML levels correlated inversely with residual liver function [30]. Elevated plasma CML levels markedly fell within 3 months after liver transplantation. This suggests that the liver also has a function in removal of plasma AGE [31]. Perhaps this occurs with the OST-48 or galectin-3 receptor, or the scavenger receptor (SR)-A or -B for all of which expression in the liver has been reported [32-34].

As for the rate of AGE removal after restoring normoglycaemia (by islet transplantation) in previously diabetic animals, the results of Sensi et al. suggest that reversal of accumulated tissue AGE takes many months to occur [35]. This may explain discrepancies between circulating and tissue AGE levels which have been reported, for example after kidney transplantation [36].

RATE OF AGE ACCUMULATION

Equilibrium levels of the reversible Schiff base and Amadori products are reached within hours and days, respectively. AGE form over longer period of weeks but remain irreversibly bound to amino groups. Using both immunohistochemical methods and four monoclonal antibodies for specific AGE molecular structures (including CML and carboxyethyllysine (CEL), but also as yet unknown epitopes), Ling et al. identified in various organs in fetal rats already at 10 fetal days with increasing accumulation during ontogeny [37]. The results suggest that CML as non-fluorescent, non-cross-linked AGE starts earlier to accumulate than fluorolink, a fluorescent, cross-linked AGE. Thus AGE accumulation starts very early, already in the fetal period. The Maillard or browning reactions that eventually lead to cross-linking and denaturation of proteins may occur with all proteins in the body, but long-lived proteins such as collagen, which represents over 30 percent of body protein, accumulate chemical damage with age.

Other long-lived proteins include lens-proteins and nerve myelin, which together with collagen are the most important targets for AGE formation. Next to these substrates other forms of macromolecules containing a free amino group are susceptible to AGE formation, like lipids and nucleic acids [38]. Especially in uraemia, associated with very high AGE levels, such shorter lived compounds as lipid constituents are affected. It must be pointed out that the dynamics of circulating AGE may be quite different: in rats with acute renal failure CML levels (measured fluorometrically) increased threefold within 48 hours [39]. The various pathways in the formation and removal of AGE are summarized in *Figure 1*.

EXAMPLES OF AGE

As mentioned before, the information on the chemical structure of AGE is limited. Of the several dozens of AGE moieties only a minority has been characterized, even less have been related to clinical human disease. In 1984 Pongor et al. first proposed a structure for AGE. They suggested the fluorescent aromatic heterocyclic compound 2-(2-furoyl)-4-(5)-(2-furanyl)-1-H-imidazole (FFI) generated through a condensation of two Amadori products [40]. They also emphasized that FFI is likely to constitute less than 1% of total AGE present in vivo. Sell and Monnier [41] identified a fluorophore present on human dura mater collagen. This fluorophore was identified as an imidazo-(4,5-b)-pyridinium ring with lysine and arginine side chains, named pentosidine. Two other pyrrole-related compounds have now been identified. These are 1-alkyl-2-formyl-

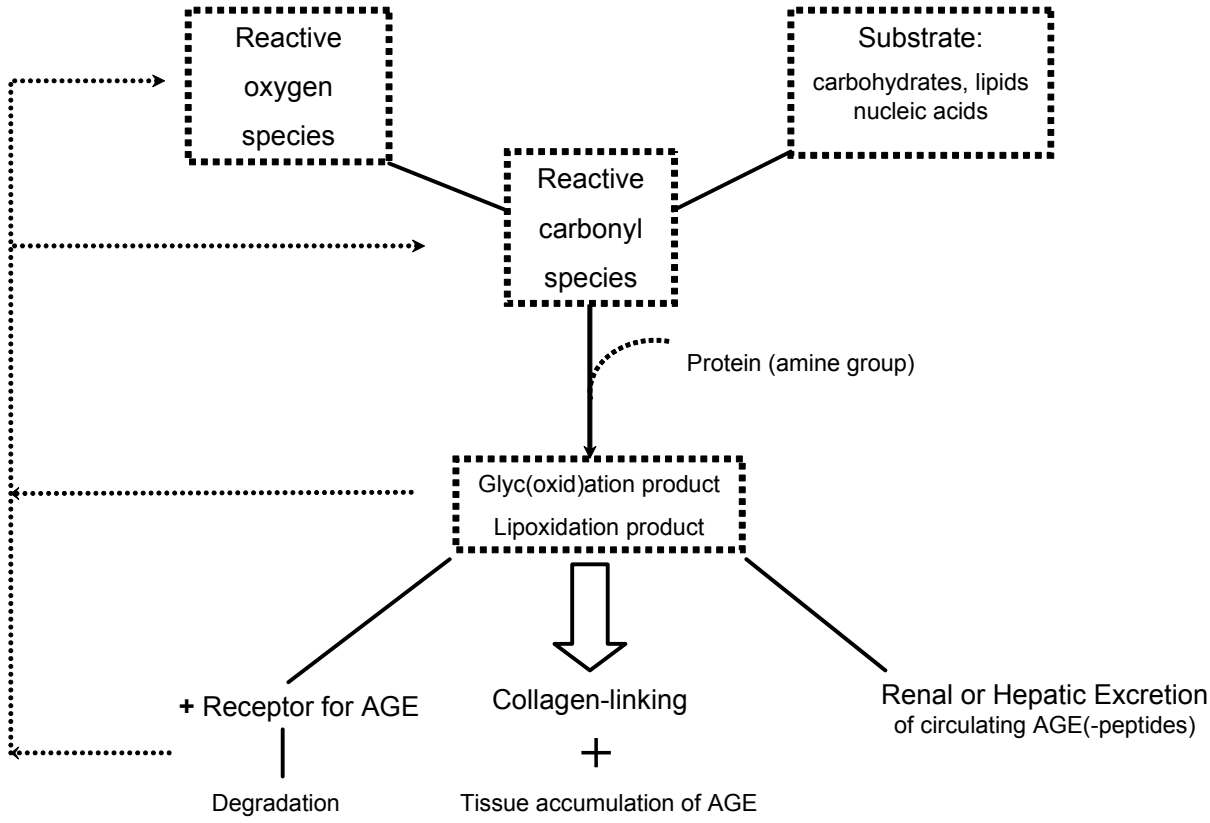

Figure 1. Steps and pathways in the formation and excretion of glyc(oxid)ation and lipoxidation products (normal lines). Products of the diverse steps in AGE formation are able to stimulate AGE formation themselves (broken lines).

3,4-diglycosylpyrrole (AGFP) and pyrraline (5-hydroxymethyl-1-neopentylpyrole-2-carbaldehyde). CML, pentosidine and hydroimidazolone are known to be glycoxidation products which means that they require oxidation reactions for their formation from glucose. As these last three compounds are well characterized they are frequently used as marker for AGE levels in plasma and in histological studies. The structure of pentosidine and CML, but also of some other AGE like pyrraline, CEL and imidazolone has been elucidated. A characteristic property of several AGE is their fluorescence. CML is, however, non-fluorescent. CML may be a dominant AGE antigen in tissue proteins [42,43]. The known AGE are immunologically distinct and coexist on different carrier proteins such as collagen, albumin, haemoglobin, lens crystalline, but also LDL cholesterol. Pentosidine and CML are well-known examples of advanced glycoxidation products, resulting from combined glycation and oxidation. In blood 90% of pentosidine and CML are bound to protein with 10% being free. Under normal conditions Hb-AGE accounts for 0.42% of circulating haemoglobin but this increases to 0.75% in diabetic subjects [44]. Hb-AGE concentration responded slowly to intensive glycemic control and may reflect glycemic control over the preceding 10-14 weeks rather than the 6-8 weeks indicated by HbA1c [45]. In early studies, using ELISA for total AGE, up to 100 fold increases were reported in serum AGE in diabetics with end stage renal disease (ESRD), in later studies 23-fold increases in serum pentosidine in ESRD [46].

Nagai et al. recently described a new precursor of CML, glycolaldehyde (GA)-pyridine, which was present in the cytoplasm of foam cells and extracellularly in the central region of atheroma in human atherosclerotic lesions [47].

BIOLOGICAL EFFECTS OF AGE

Changed Protein Function by Conformational Changes, the Role of Cross-Linking

A common consequence of AGE formation is covalent cross-linking, mostly to proteins. These cross-links may result in entrapment of proteins, lipoproteins and immunoglobulins [2]. Cross-linking of collagen proteins, for example, contributes both to the rigidity and the loss of elasticity of tissues, and increases resistance to proteolysis

[48-51]. The latter process inhibits tissue remodelling. Among the consequences are the thickening of capillary basement membrane observed in diabetes. This protein modification is also responsible for crystalline lenses becoming opaque in cataracts, a degenerative disease that is frequent in diabetic or aged persons. Glomerular sclerosis is another consequence. These processes are discussed in more detail below.

Small soluble AGE peptides released into the circulation by macrophages after degradation of AGE proteins may react and covalently bind to proteins such as collagen and LDL. Such reactive intermediates from degraded AGE can form ‚second generation, AGE and so actively contribute to the biological AGE effects.

AGE-formation on the extracellular matrix results in a decreased elasticity and increased thickness and rigidity of the vascular wall and in narrowing of the vessel lumen.

Diminished arterial elasticity in humans with diabetes was related to enhanced AGE-formation before [52]. AGE formed on vascular matrix proteins mediate defective endothelium-dependent vasodilatation by quenching nitric oxide [53]. Moreover, incubations of rat aorta segments with high-glycosylated human haemoglobin inhibited endothelium-dependent relaxation in this vessel [54]. At the microvascular level, the toxic effect of AGE on retinal capillary pericytes and endothelial cells was shown in vitro [55]. The functional effects of AGE on the endothelium include inhibition of prostacyclin production and increased plaminogen activator inhibitor 1 (PAI-1) and vascular endothelial growth factor (VEGF) production [56,57]. AGE also induce the expression of the potent vasoconstrictor endothelin-1 [58]. Part of these latter effects may be due to more effects mediated by AGE receptors than by conformational changes, as further discussed below.

Lipoprotein modification by AGE contributes to the dyslipidaemia frequently observed in diabetic patients [1]. Such a modification of LDL in diabetes may render the LDL particles more atherogenic, contributing to the atherogenic risk for diabetic patients. Another consequence may be that lipoproteins are trapped by AGE formed on the matrix components of the vascular wall, resulting in an impaired cholesterol efflux and subsequently in vascular lipoprotein accumulation [59]. This has, however, been

debated by others [60]. Glycated LDL also triggers endothelial transcription activators like STAT-5 [61].

Induction of Oxidative Stress

AGE binding to cellular binding sites results in depletion of cellular antioxidant defense mechanisms such as vitamin C and glutathione and the generation of oxygen free radicals [62,63]. These mechanisms may also involve receptor stimulation. For example, activation of NADPH oxidase in AGE-RAGE mediated generation of reactive oxygen species in human endothelial cells [64]. Semicarbazide-sensitive amine oxydase (SSAO) may have a contributory role by production of MGO [65]. Other experiments showed a potentially important role for oxygen free radicals and NO in mediating permeability and blood flow changes induced by AGE involving increased protein kinase C activity and VEGF production [66]. An additional study confirmed increased VEGF expression by AGE-albumin through the activation of hypoxia inducible factor-1 (HIF-1)[67]. In diabetes, persistent hyperglycaemia causes increased production of free radicals via the processes of autoxidation of glucose and non-enzymatic protein glycation, discussed above, and via an enhanced flux of glucose through the polyol pathway [68]. Oxidation of plasma lipids may stimulate autoxidative reactions of sugars, enhancing damage to both lipids and proteins in the circulation and the vascular wall. In this way, the cycle of oxidative stress and damage is continued and reinforced. Bucala et al. first showed the effect of advanced glycosylation on lipid oxidation [69]. Its role in accelerating atherosclerosis has been discussed by Vlassara and by Bierhaus [2,70].

Receptor Stimulation and Post-Receptor Effects of AGE

AGE formation not only changes the physiological properties of proteins and other molecules, but also induces cellular dysfunction through interaction with more or less specific cell surface receptors. An increasing number of AGE-receptors has been identified and characterized. These receptors include the receptor for AGE (RAGE),

AGE-R1, AGE-R2 and AGE-R3, but also other receptors classified as scavenger receptors (SR), SR-A and SR-B.

RAGE was identified in 1992 as a 35-kDa protein in bovine lung endothelial cells, and is still considered as a representative AGE receptor on endothelial cells. It belongs to the immunoglobulin superfamily [71,72]. In contrast to scavenger receptors, binding of AGE to RAGE does not result in endocytosis of AGE, but results by intracellular signalling in, abnormally sustained, activation of NF-kappa-B, and subsequent expression of endothelial adhesion molecules and tissue factor [73,74]. Epithelial-myofibroblast transdifferentiation, an important step in the development of tubulointerstitial fibrosis which is often observed in diabetic nephropathy, is caused by AGE-RAGE interactions [75]. Enhanced RAGE expression also has been correlated with nephropathy, retinopathy, neuropathy, autoimmune, and inflammatory disorders. In the vasculature RAGE has been associated with the induction of apoptotic death [76]. This action may be responsible for several complications related to diabetes, including vascular leakiness and nonresponsiveness. Animal studies using soluble RAGE to block the RAGE receptor also show a reduction in vascular permeability and a suppression of vascular lesion formation. RAGE antibodies were also found by Esposito et al. to prevent an increase in adhesion of peripheral blood mononuclear cells during hyperglycaemia [77]. Not only AGE but also amphoterin and amyloid beta-peptide are major ligands for RAGE. Amyloid beta-peptide is thought to be important in the pathogenesis of Alzheimer's disease; the role of AGE in this disease is discussed below. At least 9 polymorphisms of the RAGE gene exist. One of these polymorphisms (-429 C allele) has an increased prevalence in type 2 diabetes patients with compared to those without retinopathy [78]. A low expression of the AGE-R1 gene in macrophages from diabetic (type 1 like) mice, but also in fresh peripheral blood mononuclear cells and in Epstein-Barr virus (EBV)-transformed cells from type 1 diabetic patients with complications, together with elevated serum AGE levels, suggests that the normal function of AGE R1 receptor in the turnover of AGE may be ineffective in these patients, possibly by genetic influences [79]. Double transgenic mice with both overexpression of human RAGE in vascular cells, and development of insulin-

dependent diabetes had renal histological changes and increased serum creatinine and albuminuria. The development of histological and functional diabetic nephropathy was prevented with the AGE-inhibitor OPB-9195 [80]. Thus, the functional consequences of RAGE binding depend not only on AGE levels, but also on variations in gene polymorphism and expression. Galectin-3 is a lectin-like protein that has been identified as a component of p90, a 90 kDa AGE-binding protein, originally identified in the rat liver [81]. Galectin-3 modulates cell adhesion, cell cycle control, and mRNA splicing. Galectin-3 may have a protective role against diabetic glomerulopathy, as suggested by the increased AGE accumulation and accelerated glomerulosclerosis in galectin-3 knockout mice made diabetic [82].

AGE which are negatively charged have been shown to bind to SR-A type I and II. In these experiments AGE-BSA underwent active endocytosis upon binding to SR-A [24]. Remarkably, CML does not bind to SR-A while glycolaldehyde-modified proteins which generate CML do [47]. CD36, a highly glycosylated protein of 88kDa, binds various ligands such as fatty acids, collagen, oxidized LDL and has recently been shown to act also as an AGE receptor [83]. CD36 acts in adipocytes as a fatty acid transporter and in macrophages as a receptor for oxidized LDL [84]. CD36 is markedly expressed in the core of atherosclerotic lesions. CD36 is considered as an important oxidized LDL receptor. AGE-binding to this receptor may contribute to the acceleration of atherosclerotic lesions. Apart from modification of LDL-cholesterol by LDL-AGE-formation which is much slower degraded in macrophages, as discussed elsewhere in this paper, and apart from the effects on CD36 as an important receptor for oxidized LDL, AGE may also more directly affect cholesterol metabolism by binding to another scavenger receptor, the SR-B1. SR-B1 is very similar, if not identical, to the one that internalizes acetyl LDL (and oxidized LDL) [34]. SR-B1 also recognizes high density lipoprotein (HDL) and mediates its uptake [85]. SR-B1 is considered to accelerate reverse cholesterol transport by promoting cholesterol efflux from peripheral cells, for example from macrophages in the vascular wall, and mediates selective uptake of HDL-cholesterol by hepatocytes. Ohgami et al. showed that SR-B1 recognized AGE as a ligand. No cross-competition as ligands seems to

exist between HDL and AGE-binding proteins. However, intracellular processing of HDL may be affected by an interaction between SR-B1 and AGE [59]. AGE also affect SR-B1 mediated cholesterol efflux but not SR-B1 independent cholesterol efflux. Thus AGE seem to inhibit reverse cholesterol transport [86].

Expression of the different AGE receptors has been found on a wide range of cell-types including monocytes, macrophages, endothelial cells, mesangial cells, fibroblasts, smooth muscle cells and tubule cells [64,74,79,87].

MARKERS OF AGE AND PROBLEMS WITH CURRENT ASSAYS

Because the structure of AGE is complex and heterogeneous, measurement of tissue and serum AGE has been difficult, and, as there is no recognized standard, different groups may not be measuring in exactly the same way. Reproducibility of many assays is rather poor. The limited knowledge on the specific structure of different AGE moieties also makes it difficult to draw any firm conclusions on the relative toxicity of different specific AGE. *Table 1* summarizes the different markers of AGE and their advantages and disadvantages. Classically fluorescence is the method used in many clinical studies as a marker of AGE. AGE has a yellow brownish pigmentation with a characteristic fluorescence pattern: excitation in the range 350-390 nm and fluorescence emission at 440-470 nm. Applying fluorescence spectroscopy, AGE have been measured in serum, urine and also in different kinds of tissues [88-90]. The presence of other, non-AGE, fluorescent substances like NADPH and of non-AGE protein adducts, such as glucose- or lipid-derived oxidation products, that exhibit similar fluorescence spectra, as well as the interference by non-protein tissue components, makes the specificity of this method low. Fluorescent quenching associated with biological samples may also impede specificity. Furthermore, many but not all AGE have fluorescent properties. Examples of non-fluorescent AGE are CML and pyrraline [91,92]. The fact that most clinical data on the relation with AGE accumulation have still been based on association with fluorescence makes this method still valuable. However, tissue samples are required, which makes this invasive

Table. 1. Markers of AGEs and their methods. The advantages and disadvantages of the different AGE assessment methods are presented in the third and fourth column of the table

Method	Marker	Compartment	Advantages	Disadvantages
Fluorescence	Collagen linked fluorescence (CLF): excitation 370 nm, emission 440 nm	tissue samples (skin, kidney, cartilage)	proven association with (diabetic) complications	-invasive, thus not feasible for frequent AGE monitoring -interference of non-AGE fluorophores, no detection of non-fluorescent AGE
	Autofluorescence: excitation 360-370 and emission 420-600 nm	in vivo skin	non-invasive, rapid method, therefore feasible for repetitive AGE assessments	-clinical relevance not proven yet -interference of non-AGE fluorophores, no detection of non-fluorescent AGE
HPLC	Pentosidine, CML, etcetera	plasma (tissue)	little invasive, practicable for AGE monitoring	-time consuming, expensive -correlation with tissue AGE-accumulation unclear -only applicable to AGE with known biochemical structures
	Hb-AGE	plasma	little invasive, practicable for AGE monitoring	-correlation with tissue AGE-accumulation unclear, because of: -insufficient representative for AGE bound to other proteins -relative short half-life of hemoglobin
ELISA	Pentosidine, CML, etcetera	serum, urine (tissue)	little invasive, low-cost, practicable for AGE monitoring	-correlation with tissue AGE-accumulation unclear -low sensitivity -low reproducibility between different laboratories
Collagen croslinking	protein cross-linking index	tissue samples	provides an indirect quantitative measure of tissue AGE accumulation	-low specifity -only valid to collagen rich tissue -reproducibility unclear
	differential scanning calorimetry	tissue samples	quick quantitative method for assessment of in vitro collagen crosslinking, also applicable in tissues with less collagen	-insufficient specifity for in vivo application, therefore clinical relevance unlikely

technique less suitable for repetitive AGE monitoring. Recently, Meerwaldt et al. reported on a rapid, non-invasive measurement of skin autofluorescence to asses AGE accumulation [93]. Although the above mentioned limitations of fluorescence still are valid, this non-invasive technique is much more convenient. If a relation with clinical data and its prognostic value are confirmed, this method might offer a feasible method in patient care.

AGE with known structures, such as pentosidine and CML, can be measured with a high degree of specificity using HPLC [94,95]. More sophisticated methods like GCMS and LC-MS became recently available for the determination of AGE. These methods are, however, quite time-consuming, require considerable training and, are thus expensive. Furthermore, the AGE with a known biochemical structure might only account for a small portion of the total amount of circulating and tissue AGE. Whether the AGE with known structure are clinically relevant is not sure.

Less time intensive, cheaper and more feasible for clinical use, are immunoassay methods, using antibodies against AGE. Nakayama first produced and characterized antibodies raised against keyhole limpet haemocyaninebound AGE [96]. The result was a polyclonal anti-AGE antiserum that was used in an ELISA. Also, an immunochemical detection of AGE in vivo was developed using antibodies raised against bovine serum albumin (BSA)-AGE or RNase-AGE [97,98]. In this way, AGE could be detected that were immunochemically different from characterized AGE such as CML, pentosidine and pyrraline. Thus far it remains uncertain whether these antibodies can identify all AGE produced in vivo. A common structure in AGE modified macromolecules that interacts with polyclonal antibodies has been suggested [97]. But, polyclonal antibodies against AGE-BSA or AGERNase failed to recognize CML which is claimed to be a dominant AGE in human serum albumin and in tissue proteins [99]. Another problem may be that factors in human serum interfere with the measurement [100]. Roche Diagnostics (Penzberg, Germany) has developed a commercially available competitive ELISA, using an anti-CML monoclonal antibody 4G9 [101,102].

Although many different antibodies have been produced, reproducibility and sensitivity remains a substantial problem. This is probably due to a lack of a well-defined set of antigens. Before antibody assays can be used as a standard, reproducible synthesis of well-defined antigens should be attained. A recent report demonstrated that shortterm heating, as used in the heat-induced epitope retrieval technique in formalin-fixed, paraffin-embedded tissue sections, results in artificial AGE formation and profoundly affects CML formation. This may also have served as an artefact in immunohistochemistry studies [103].

A competitive radioreceptor assay based on AGE-specific receptors on the macrophage-like tumour cell line RAW 264.7 was developed earlier, but is also not widely used, possibly due to the strong interference in this assay with polyanions such as heparin [104].

As an indirect measure of AGE, collagen crosslinking has been proposed. The extracellular matrix protein collagen is highly prone to AGE-formation. It has been shown that the susceptibility of collagen to digestion by pepsin provide an index of protein cross-linking [91,105]. In this method, often tissues are used with high amounts of collagen, like rat tail tendon, to increase specificity of the measurement. Thus, this method only provides an indication of tissue AGE accumulation. Moreover, some AGE such as CML and pyrraline do not occur as crosslinks in proteins. Applying differential scanning calorimetry (DSC), crosslinking can also be determined in tissues with less collagen. Although this method will provide a quantitative method for determining the crosslinking of collagen in vitro, it lacks the specificity to assess crosslinking in vivo. Mentink et al. showed that DSC was able to detect a lower rate of crosslinking by aminoguanidine in rat tendons (but not in rat skin) [106]. However, no effect of the AGE breaker ALT-711 was found in either tissue with DSC. The advantage of DSC is that it is quick and can be well quantified in comparison with methods like enzyme digestibility.

Miyazaki recently attracted attention to the problems associated with preparing AGE-BSA (bovine serum albumin) complexes that are used in ligand experiments for scavenger receptors. Their preparations belong to the extensively modified preparations

in comparison to those from some other laboratories [86]. Marked differences in ligand activity to scavenger receptors may exist even when specific AGE like CML are present in both of them. They suggested that the presence of glycolaldehyde may be important for binding behaviour of AGE preparations to receptors.

Subramaniam warned against the widely used dichlorofluorescein assay to assess cellular oxidant stress by microtiter plate assay because of MGO effects [107]. Hui et al. found that trace amount of redox-active metal ions in biological buffers may induce oxidative stress and alterations in cellular functions which would otherwise be possibly ascribed to AGE-proteins [108].

AGE IN DISEASE

A dynamic equilibrium seems to exist between AGE circulating in blood, AGE accumulating in tissue and renal (and perhaps hepatic) clearance of AGE. It is no surprise that AGE accumulation exceeds normal in patients with diabetes mellitus or renal failure [109-112]. The association with various chronic complications of these diseases is discussed below in more detail. AGE have also been reported to accumulate in amyloid associated diseases such as dialysis related amyloidosis and Alzheimer Disease, and in the plaques found in atherosclerosis. In the neurological field increased local AGE accumulation has also been reported in amyotrophic lateral sclerosis. Accumulation of AGE in cartilage has been linked to synovial inflammatory diseases, including rheumatoid arthritis and osteoarthritis. *Table 2* shows an overview of all diseases discussed in this paragraph with related references.

AGE Accumulation in Diabetes Mellitus (DM) and Chronic Complications

Increased concentrations of AGE have been associated with chronic complications in cross-sectional studies in DM: AGE (CML, pentosidine, others) content of skin collagen has been shown to be a risk marker for microvascular complications of diabetes, independent of actual glucose or recent HbA1c values [113]. Serum AGE are increased in diabetic patients compared to controls [46,114,115]. AGE concentrations in serum, but also in kidneys, skin and vascular tissue may rise within 5 weeks after

Table 2. Overview of diseases or complications in which AGE accumulation has been described in the literature to play a pathogenetic role.

Diseases with increased AGE accumulation			References
Diabetes Mellitus			
		Cardiovascular disease	35, 47,116, 120, 167
		Retinopathy, cataract	6, 121-132
		Nephropathy	46, 80, 90, 121, 133-136
		Neuropathy	137-142
		Periodontitis	191, 192
Renal failure			
	Peritoneal dialysis		3, 147, 151
	Hemodialysis		3, 150, 152-156
	Kidney transplantation		36, 163-165
		Dialysis related amyloidosis	76, 157-159
		Disturbed bone metabolism	160, 161
Atherosclerosis			109, 114, 166-174
Alzheimer disease			175-182
Amyotrophic lateral sclerosis			183, 184
Arthritis (Osteoarthritis, Reumatoid arthritis)			185-187
Amyloidosis			188-190

making an animal diabetic, for example with STZ [96]. Serum AGE levels are higher in both type 1 and type 2 DM, are already increased early in the disease, as shown in prepubertal diabetic children to approximately 1.5-fold, and are associated with the severity of retinopathy and nephropathy, but also with coronary heart disease in type 2 diabetes mellitus patients [116]. Hb-AGE rises from 0.42 to 0.75% in diabetic subjects [44]. In patients with DM combined with ESRD serum AGE levels were increased 100-fold compared to diabetics with normal renal function, in another study serum pentosidine was increased 23-fold in DM with ESRD [46,117]. Comparable rank orders for subjects with DM and ESRD, DM without renal disease and controls have been reported for arterial wall AGE content [46]. Vascular wall AGE content has been semi-quantitatively found to be increased in subjects with DM and vascular disease, being present in fatty streaks and macrophages [118].

When exogenous AGE are administered to rats to attain plasma levels equivalent to those in diabetic rats the renal AGE content was 50% greater than in control rats after 5 months, accompanied by a glomerular sclerosis with a 50%

expansion of the glomerular volume, and with an increase in mesangial matrix and capillary basement membrane thickness [119].

Sensi et al. investigated the effects of restoring normoglycaemia 4 months after homologous islet cell transplantation in different organs of rats previously made diabetic shortly or for longer periods before transplantation [35]. Only after a longer (8 months) preceding period of DM AGE content was elevated in all organs. In the eye lens but not in the aorta AGE content was lower than in nontransplanted diabetic control rat. This suggests that in DM prolonged time periods are an essential prerequisite not only for the accumulation of AGE to develop, but also to be reversed.

In addition to the earlier discussed biological effects, AGE accumulation may also contribute to dyslipidaemia in diabetes both by affecting the HDL receptor and by AGE-modified LDL [1]. The modification of LDL in diabetes may render the LDL particle more atherogenic, thereby increasing the atherogenic risk for diabetic patients. Park and co-workers showed that administration of the soluble extracellular domain of the receptor for AGE (RAGE) completely suppressed diabetic atherosclerosis in STZ-diabetic apolipoprotein E-deficient mice, in a manner independent of the level of glucose and lipids [120].

Diabetic Retinopathy and Cataract

Background diabetic retinopathy has developed after 25 years in almost all patients with DM, a much lower percentage has then developed proliferative retinopathy (8 to 26%). CML and pentosidine skin collagen accumulation is associated with the severity of retinopathy in type 1 diabetes patients, independent of age and diabetes duration [6]. In an autopsy study CML was present around retinal blood vessels in diabetics, but not in controls. Furthermore it is shown that formation of AGE measured by ELISA in skin biopsies are preceded and correlated with early manifestations of retinopathy as well as nephropathy [121]. Immunohistochemical studies in rats have revealed that AGE (CML) reactivity was increased in diabetic retinas [122-124]. No difference was present between subjects with DM with proliferative retinopathy and those with background retinopathy [125]. In two diabetic animal studies the AGE formation

inhibitor aminoguanidine (see below) prevented microaneurysm formation, pericyte loss, and the development of accelerated diabetic retinopathy, respectively [126,127]. Also the development of DM-related abnormalities of the retina could be prevented by aminoguanidine, indicating a prominent role for AGE in this complication [122].

Glycation of lens crystalline, and subsequent oxidation, may play a major part in accelerated cataract formation in DM. Alterations in Na+/K+-ATPase activity due to glycation may contribute to this. Evidence for AGE formation has also been found in the vitreous body using an AGE-specific ELISA, and was found to be related to age and the presence of DM [128]. In the vitreous body of patients with diabetic retinopathy and in a nondiabetic control group the increased levels of pentosidine in diabetics are strongly related to VEGF and other cytokines [129]. AGE accumulation has also been observed in the cornea of diabetic patients [130-132]. The formation of AGE on fibronectin and laminin in Descemet's membrane in the cornea of the eye inhibited the attachment and spreading of endothelial cells . This may contribute to the increased prevalence of corneal endothelial abnormalities in patients with DM [131]. Thus, several lines of evidence indicate a role for AGE in the development of several forms of diabetes related eye disease, especially retinopathy, with a favourable effect of aminoguanidine.

Diabetic Nephropathy

Diabetic nephropathy develops in approximately 40% of patients with DM. Multiple studies have shown that AGE are important factors in the pathogenesis of nephropathy. CML, pyrraline and pentosidine have all been found in increased quantities in kidneys of patients with DM with or without ESRD [133]. AGE accumulation is related to the severity of diabetic nephropathy. Animal studies in rats have immunohistochemically shown AGE accumulation in the glomerular extracellular matrix. Expression of proteoglycans which form an important constituent of the kidney extracellular matrix is decreased in cultured kidney epithelial cells at CML-protein concentrations as found in diabetes [134]. In fact, the hyaline depositions in the classical Kimmelstiel-Wilson lesions of diabetic kidneys consist for a major part of AGE. Exposition to AGE-

crosslinked proteins results in increased oxidative stress in rat mesangial cells and in an increase in protein kinase C activity. Makita et al. showed that the increase in circulating AGE-peptides of diabetic patients correlates with the severity of renal function impairment [46]. Deuther-Conrad highlighted the toxic effect of the AGE-peptides in cultured kidney epithelial and proximal tubular cells [135]. Overexpression of RAGE in transgenic diabetic mice resulted in histological and functional changes of diabetic nephropathy [80].

Pharmacological inhibition of AGE-formation by aminoguanidine prevented the development of kidney lesions, albuminuria and mesangial expansion in diabetic rats [90,136]. In human studies discussed in more detail below, aminoguanidine 150 mg twice a day reduced proteinuria significantly in a large (690 patients) study in patients with type 1 DM and moderate renal impairment. As mentioned before, Beisswenger and colleagues showed that formation of AGE measured by ELISA in skin biopsies preceded and correlated with early manifestations of retinopathy as well as nephropathy [121].

Diabetic Neuropathy

Using sensitive methods the large majority of patients (80-90%) with DM will eventually develop symptoms or signs of diabetic polyneuropathy, a much smaller percentage develops autonomic neuropathy. The important role of glycation in the development of diabetic neuropathy has been recently reviewed [137]. In vitro, diverse mechanisms involving AGE in neuropathy seem to play a pathogenetic role: accumulation of AGE in vasa nervorum results in wall thickening, occlusion and ischemia of nerves, and consequent myelin damage with segmental demyelination. Glycation of the axonal cytoskeletal proteins such as myelin and neurofilament results in slowed axonal transport and axonal degeneration [138]. Nerve fiber regeneration may be reduced because the function of nerve growth factor and other growth factors like fibrin, but also because laminin in Schwann cell basal laminas is glycated and impeded in its function [139]. In animal studies aminoguanidine prevented AGE-induced reductions in peripheral sensory nerve conduction velocity and action

potentials [140,141]. In diabetic humans more CML is found in the perineurium, in pericytes of endoneurial microvessels and in both myelinated and unmyelinated fibers, with a correlation between CML accumulation and myelinated fiber loss [142].

Furthermore, aminoguanidine treatment reduced experimental diabetic neuropathy, as shown by a significant improvement of the reduced motor nerve conduction velocity, partially through the correction of the endoneurial microcirculation [141].

Taking all this together, considerable evidence exists supporting that AGE contribute prominently to the development and exacerbation of diabetic neuropathy.

AGE in Renal Insufficiency, Renal Replacement Therapy and Kidney Transplantation

Accumulation of reactive carbonyl compounds (RCO) and AGE is markedly increased in plasma, serum and tissues of diabetic and non-diabetic patients with ESRD [143-145]. This has also been shown by Meng et al. for CML levels in plasma and skin in patients with chronic renal failure (CRF) both with and without diabetes [146]. Thus, hyperglycaemia is not a prerequisite for AGE accumulation. High serum AGE levels are also found in patients on hemodialysis (HD) or continuous ambulatory peritoneal dialysis (CAPD), probably by the introduction of high concentrations of glucose in dialysis fluids in CAPD (74-214 mM) [3,147]. Impaired clearance of AGE and AGE precursors is responsible for this increased AGE accumulation. Although direct evidence is insufficient, increased oxidizing conditions and the development of reactive oxygen species might also accelerate the formation of AGE [8,148,149]. Polymorphonuclear leukocyte activation by uremic toxins and interaction with the dialysis membrane in HD facilitate this. Diminished clearance of low molecular weight precursors of AGE and diminished catabolism of AGE precursors may further contribute to tissue AGE accumulation in CRF [3,150]. Lal et al. addressed the question of AGE-dependent signaling in the mesangium and found evidence for an important role of oxidative stress [149]. HD clears only the free and not the protein bound forms of AGE. CAPD is associated with lower plasma pentosidine levels, possibly by clearance of albumin-bound pentosidine in the peritoneum [3]. In rats

structural and permeability changes of the peritoneum with increased nitric oxide synthase expression are found associated with vascular deposits of AGE in chronic uraemia [151]. As for the relation with cardiovascular complications in renal failure, remarkably Schwedler et al. showed that circulating AGE levels (total fluorescent AGE, and CML, both in serum) do not predict mortality in HD patients [152]. Plasma pentosidine as an AGE and marker of ,carbonyl stress, was neither found to be related to intima media thickness or atherosclerotic plaques in the carotids in HD patients [153]. Schwedler et al. proposed that the benefit of high serum AGE levels might reflect a better nutritional support, which improves survival. However, an alternative explanation is that plasma or serum AGE levels may not adequately reflect tissue AGE accumulation. Floridi et al. found that a shift from standard dialysis regimen 3 times a week to daily HD resulted after 6 months in substantially lower plasma AGE levels (pentosidine and two AGE peptides), but did not measure AGE in other tissues [154]. High concentrations of glucose (and alpha-oxoaldehydes) in dialysis fluids also increase glycation. Vitamin-E coated dialysis filters prevented the rise in serum AGE levels found with conventional polysulfone filters. After six months of HD with these vitamin E coated filters, the pre-dialysis levels of AGE and other markers of oxidative stress were reduced [155]. Very high flux polysulfone filters reduced pre-dialysis serum values of free and protein-bound pentosidine and AGE peptides and beta2-microglobulin (B2-MG) [156]. Several different AGE have been detected in B2-MG amyloid deposits arising after chronic renal HD [157]. Amyloid deposits are found in bone, articular cartilage, synovium, muscle and ligaments, giving rise to shoulder periarthritis, carpal tunnel syndrome and flexor tendosynovitis. AGE-modified B2-MG induces chemotaxis of monocytes and the production of cytokines (interleukin (IL) 1b, IL-6 and TNFa). AGE-modified B2-MG is cleared less than native B2-MG through dialysis membranes [157]. Hou et al. showed that the local inflammatory response to B2-MG amyloid deposits by monocytes/macrophages which is a characteristic histological feature of dialysis-related amyloidosis (DRA), is dependent on delayed apoptosis of monocytes by AGE-modified B2-MG [76]. These cytokines may stimulate local production of B2-MG and bone destruction. RAGE expressed by synovial

fibroblasts may mediate some of these responses involved in DRA [158]. CML-haemoglobin has been proposed as a possible predictor of progression of bone cysts in DRA [159]. Thus, at least a close relation between AGE accumulation in renal failure and the development of dialysis related amyloidosis exists. As for the possible osseous sequela of AGE accumulation, it should be added that pentosidine is related inversely to parathyroid hormone (PTH) and bone alkaline phosphatase in HD patients. The authors found this to be in agreement with AGE accumulation as a factor in reduced bone turnover in dialysis patients [160]. Yamamoto also found that AGE-BSA in vitro inhibits osteoblastic activity and inhibits PTH secretion in response to hypocalcaemia [161].

Renal transplantation is considered to be the best therapy for lowering AGE in patients with renal failure. Although a dramatic reduction in plasma AGE levels following transplantation has been found, the levels remain higher compared with the general population [162]. Studies to date, investigating the influence of kidney transplantation on tissue AGE accumulation show contradictory results [36,163-165]. While most studies report a reduction of about 50%, the only prospective study showed no significant reduction in tissue AGE. Despite the improvements in immunosuppression and an excellent short-term transplant survival in kidney transplantation, 10-year graft survival remains relatively poor with 50-60% (chronic renal transplant dysfunction, CRTD) while cardiovascular morbidity and mortality also remain high, and are associated with an increased prevalence of cardiovascular risk factors. Perhaps persistently high levels of AGE in tissues, enhanced by sustained oxidative stress, contribute to the development of CRTD. When AGE are administered to healthy rats, CRTD-like lesions develop in the kidney [20]. At present, no data is available on the long-term course of plasma and tissue AGE in human CRTD.

AGE ACCUMULATION IN OTHER DISEASES

Atherosclerosis

The accumulation of AGE in the vessel wall has also been related to lipoprotein metabolism, as discussed above, and to the development of atherosclerosis [166]. AGE

accumulation has been found by immunohistochemistry on the extracellular matrix proteins and within macrophage- and smooth muscle cell-derived foam cells in atherosclerotic plaques [114,167]. Normolipidemic and euglycaemic subjects with atherosclerosis had increased AGE in the apolipoprotein B100, as did patients with uraemia [1,118]. It was claimed that AGE-modified LDL had decreased clearance from circulation and metabolism but this was a characteristic of LDL glycated to higher extent than found physiologically [168]. The expression of the RAGE and scavenger receptors were increased in atherosclerosis [169]. Interaction of AGE-modified proteins with AGE receptors may stimulate cytokine and growth factor production that sustains the development of the atherosclerotic plaques [109,170-172]. Atherosclerotic lesions in apoE knockout mice that were accelerated under STZ-induced diabetic conditions were significantly suppressed by intraperitoneal administration of a soluble form of RAGE [120]. The process of atherosclerosis may also stimulate local AGE formation. Activated macrophages in the intima make the local environment of atheroma oxidizing, which may increase AGE formation. If glycation of LDL stimulated its oxidation in vivo then glycation would be a critical initiating factor in macrovascular disease. Currently, however, there is no agreement on this, although glycated collagen stimulated peroxidation of membrane lipids in vitro [173,174]. Other factors influence LDL oxidation - antioxidant and triglyceride content, for example - and it is not yet clear how important glycation is compared to these. Once atherosclerosis is initiated, however, increased formation of AGE within the locus of the plaque and induction of AGE receptor expression is expected to sustain plaque expansion.

Alzheimer's Disease (AD)

AGE accumulation is found in senile plaques and neurofibrillary tangles of persons with AD [175-177]. In the cerebrospinal fluid of patients with AD increased levels of Amadori products on all the major proteins are found. This may contribute to increased AGE deposition in the brain of AD patients [178]. Although Meli et al. reported increased serum pentosidine levels, using HPLC, in patients with AD, so far no other

evidence exists for systemic increases of AGE levels [179]. In contrast, chromatographic analysis of acid hydrolysates of brain frontal cortex for CML and pentosidine showed no significant increase in AD subjects, relative to controls. Pyrraline and pentosidine were not detected in beta-amyloid or apolipoprotein-E extracted from brain tissue of AD subjects, and immunohistochemistry with an anti-AGE antibody showed no staining of senile plaques [180,181]. Data on RAGE expression and binding in AD are also controversial. Thus, the role of AGE in AD is, therefore, currently uncertain. It has been proposed that reactive carbonyls are implicated as neurotoxic mediators of oxidative damage in the progression of AD and so may result in AGE accumulation [182].

Amyotrophic Lateral Sclerosis (ALS)

In sporadic ALS, CML as a marker of lipid peroxidation or protein glycoxidation and pentosidine as a marker of protein glycoxidation are increased along with markers of lipid peroxidation immunohistochemically in the spinal cord in almost all motor neurons and in reactive astrocytes and microglia/macrophages [183]. The authors implicate the formation of CML, pentosidine or lipid peroxidation products in motor neuron degeneration. A special familial form of ALS exists with superoxide dismutase-1 (SOD-1) gene mutations, characterized by the presence of neuronal Lewy body-like hyaline and astrocytic hyaline inclusions. Ultrastructurally these inclusions contain granule-coated fibrils positive for a SOD-1 protein but also modified by AGE. The formation of AGE-modified mutant SOD-1 is probably one of the mechanisms responsible for the fibril formation [184].

Arthritis

Drinda et al. detected CML in the synovial tissue in patients with osteoarthritis and also rheumatoid arthritis [185]. Adverse changes induced by AGE in the collagen network in bone are associated with a loss of toughness of bone in elderly people [186]. De Groot et al. found in in vitro experiments with cartilage from osteoarthritis or rheumatoid arthritis patients that increased cartilage AGE reduced cartilage

degradation by matrix metalloproteinases (MMP) in synovial fluid, suggesting that aged cartilage is less sensitive to the MMP-mediated cartilage destruction that occurs in osteoarthritis and rheumatoid arthritis. This may have effect on their rate of progression [187].

Amyloidosis

In various types of amyloidosis abnormal amyloid fibril proteins are associated with AGE accumulation [188]. Familial amyloidotic polyneuropathy (FAP), Portuguese type, is caused by amyloid depositions of a mutated transthyretin, and is associated with gastrointestinal symptoms like diarrhea, malabsorption due to severe dysmotility of the gastrointestinal tract. RAGE has been implicated in amyloid toxicity, and transthyretin amyloid fibrils have been shown to have affinity for RAGE and to induce NF-kappaB activation and apoptosis (also see above for RAGE effects). In autopsy samples immunoreactivity for AGE and RAGE was found at the same sites as amyloid deposits and transthyretin [189,190].

Periodontitis

AGE present in diabetic gingiva may be associated with a state of enhanced oxidant stress, a potential mechanism for accelerated tissue injury [191]. The degree of collagen crosslinking has a marked influence on bone regeneration of calvarial defects in rat studies. This may suggest a negative role of AGE-crosslinked collagen on recovery from periodontitis [192].

INTERVENTIONS

Different approaches may be used to counter AGE accumulation and its deleterious effects. The first approach is to reduce the formation of AGE at one of the many steps involved. This ranges from interventions aimed at reducing glucose levels in diabetes to drugs specifically aimed at one of the late steps in the Amadori process. Recent studies of the group of Vlassara et al. address the possibility to reduce AGE accumulation by limiting the exogenous supply of AGE from food. Since smoking is

another source of exogenous AGE and associated with increased AGE accumulation, therefore smoking cessation is another candidate in this category. The second approach is to increase breakdown of AGE. One drug, ALT-711, is now in advanced phase II studies as an AGE-breaker. The third approach aims to prevent the deleterious effects of AGE. It includes interventions aimed at reducing protein cross-links, or at blocking or competing for one or more of the different AGE receptors (summarized in *Table 3*).

Table 3. Interventions to reduce AGE accumulation.

Inhibition of AGE formation	
	Specific: aminoguanidine, OPB-9195, ALT-946, pyridoxamine (or other "Amadorins")
	Aspecific: metformine, cerivastatine, ACE-inhibitors, ATII-antagonists, other pharmaceuticals that reduce oxidative stress
AGE breaker	
	ALT-711
AGE-RAGE interaction blockers	
Reduction of exogenous AGE	
	ceasing tobacco use, low-AGE diet

Inhibitors of AGE Formation

As discussed above AGE formation is a complex process and under the influence of diverse mechanisms like glycation and oxidative stress. This offers many opportunities to prevent AGE formation. In fact, any treatment aimed at lowering glycation or oxidative stress may be considered. Usually, however, attention is focused more on interventions aimed at one of the steps in the Amadori process directly preceding AGE formation. The drug attracting most attention has been aminoguanidine.

Aminoguanidine. In 1986 Brownlee et al. showed that aminoguanidine prevented both the formation of fluorescent AGE and the formation of glucose-derived collagen cross-links in vitro [193]. Furthermore, administration of the drug to diabetic rats prevented fluorescent AGE-formation and cross-linking of arterial wall connective tissue protein in vivo. Aminoguanidine reacts with Amadori-derived reactive intermediates such as 3-deoxyglucosone. Incubation of model Amadori products with aminoguanidine produces a triazine compound which prevents the further rearrangement of intermediates to protein-protein and protein-lipid crosslinks [194]. Price et al. addressed the chelating activity of aminoguanidine and other AGE inhibitors (carnosine, OPB-9195) and breakers (ALT-711). They conclude that rather

than by trapping reactive carbonyl intermediates they act primarily as chelators and antioxidants [195]. Aminoguanidine does not interfere with already formed AGE. Aminoguanidine also reduces NO formation through inhibition of inducible NO-synthase. Several studies claim that aminoguanidine selectively inhibits NO-synthase, probably both the constitutive and the inducible form [196-200]. Furthermore, aminoguanidine may act as a free radical scavenger and so reduce oxidative stress [201,202]. Many studies have addressed its use in preventing diabetic micro- and macrovascular complications. The beneficial effect of aminoguanidine is considered by most authors to be mainly due to its direct effect on AGE formation. However, dissociations between reductions in albuminuria and effects on skin AGE formation have also been described [203]. Studies in diabetic animal show that aminoguanidine treatment prevents or reduces the major long-term complications of diabetes, nephropathy, neuropathy and retinopathy. Reduction of AGE accumulation, albuminuria and kidney lesions were established [90,136,204,205]. It is remarkable that the lesions at the tubulointerstitial level are improved by aminoguanidine [206,207]. Increase in motor nerve conduction velocity, partially through correction of endoneurial microcirculation, normalization of nerve blood flow and improvement of nerve conduction were also found [141,208,209]. Diabetic retinopathy was inhibited by aminoguanidine treatment, with prevention of abnormal endothelial cell proliferation and microaneurysms, and reduction in pericyte dropout, even when in one of these studies no effect of aminoguanidine on AGE (pentosidine in aorta and tail collagen, Hb-AGE) [122,209]. Clinical studies with aminoguanidine or its generic form, pimagedine, are still in progress.

In addition, aminoguanidine interferes with the development of atherosclerotic lesions: increasing elasticity and decreasing fluid filtration in large arteries, decreasing levels of total cholesterol, triglycerides and LDL-cholesterol in diabetic patients treated with aminoguanidine [210].

Outside the field of diabetes aminoguanidine reduced aortic pulse wave velocity in non-diabetic rats. Aminoguanidine seems to prevent the age-related increase in wall stiffness, although no effects on wall stress or composition were found [211].

As for toxicity, a small percentage of patients develops flu-like symptoms. During longer use, macrocytic anaemia is found in a considerable percentage of patients and antinuclear cytoplasmic antibodies may develop at high doses, which is clinically associated with crescentic glomerulonephritis in three cases. Further side effects in human trials included nausea and headache. The daily dosage of aminoguanidine is usually 300 mg a day, taken with food. Aminoguanidine has a half life of only 4-hours, so these dosages would be best administered over the day into split doses. Checks of full blood counts and autoimmune antibodies are advised before and during treatment.

Other aminoguanidine-like compounds, without an influence on NO-synthase, have recently been described. They exhibit similar effects as aminoguanidine: 2,3 diaminophenazine (NNC39-0028) attenuated the development of diabetic mesenteric hypertrophy [212]; Oturai et al. found in STZ diabetic rats improved collagen solubility, in contrast to the lack of effect of the AGE-breaker PTB (see below). However, the increased urinary albumin excretion and the increased leakage in the eye of labeled albumin were not reduced by either drug [213]. 4-amino-3-hydrazino-5-isopropyl 4H-1,2,4-triazole and 3,5-diamino-4-hydroxy-benzoic acid dihydrochloride increased tail tendon collagen solubility in treated rats [105]. OPB-9195 [(+/-)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yla cetanilide] inhibits both AGE and advanced lipoxidation endproducts (ALE) formation probably by its ability to trap reactive carbonyl compounds like GO, MGO and 3-DG [214]. It improved the delayed motor nerve conduction velocity in diabetic rats, and reduced the expression of immunoreactive AGE in the nerves [215]. ALT-946 was more potent than aminoguanidine in diabetic rats in suppressing immunohistochemically measured AGE production after 32 weeks. Albuminuria was equally reduced in the three groups treated from week 0 to 32 with aminoguanidine, or from week—32 or 16-32 with ALT-946 [216]. In a transgenic rat model with angiotensin II dependent hypertension, along with STZ-induced diabetes and severe diabetic nephropathy ALT-946 and aminoguanidine both reduced glomerulosclerosis and glomerular AGE immunolabeling. ALT-946 but not AG also reduced cortical tubular degeneration and albuminuria. Thus even with a strong hypertensive stimulus for developing

nephropathy in diabetic rats AGE formation inhibitors may protect against diabetic nephropathy [217].

Pyridoxamine inhibits formation of AGE derived from the alpha-dicarbonyl compound MGO in vitro [218]. It also reduces formation of lipoxidation end products (ALE). It has this capacity at least in part by trapping reactive carbonyl intermediates in the formation of AGE and ALE [219]. In diabetic rats pyridoxamine prevented after 29 weeks the development of structural changes of diabetic retinopathy and CML accumulation in the retinal vasculature [220]. Diabetic nephropathy is also inhibited by pyridoxamine to a similar extent as aminoguanidine in STZ-diabetic rats, associated with a marked reduction of AGE/ALE, CML, CEL, cross-linking and fluorescence in skin collagen [221]

In 1999, a novel group of inhibitors of the conversion of Amadori intermediates to AGE, named "Amadorins" was described [222]. The therapeutic potential of these drugs is currently being investigated. Other agents such as Carnosine and Acetyl-L-Carnitine also prevent AGE formation. Carnosine (beta-alanyl-lhistidine) is physiologically present in muscle and nervous tissues in many animals, especially long-lived species [223]. As yet only in vitro studies found that N-acetylcysteine suppressed CML production during prolonged incubations [224].

In a recent study it was demonstrated that ACE inhibition with ramipril and aminoguanidine after 12 weeks equally reduced renal AGE accumulation, assessed by immunohistochemistry and serum and renal fluorescence, in STZ-induced diabetic animals. Both drugs prevented diabetes-induced increases in expression of RAGE and also AGE R2, R3 receptors and NFkappaB. The link between inhibition of the renin-angiotensin system and of AGE accumulation may reside in the reduction of oxidative stress [225]. The inhibition of AGE production by interventions in the renin-angiotensin system is confirmed by an in vitro study by Miyata et al. for the angiotensin II type 1 receptor antagonist olmesartan and the ACE-inhibitor temocaprilat. In a comparison with nifedipine, both olmesartan and temocaprilat inhibited the formation of pentosidine and CML [226]. An in vitro study with different

calcium antagonists showed some antioxidative properties of calcium antagonists but no inhibitory effect on the formation of AGE precursors [227].

Beraprost sodium, a prostacyclin analogue and cyclic-AMP-elevating agent, was found to protect retinal pericytes with prevention of AGE-induced apoptotic cell death from AGE-induced cytotoxicity through its antioxidative properties [228].

AGE-Breakers

In 1996, Vasan and colleagues described a new compound that cleaves glucose-derived protein cross-links in vitro and in vivo [229]. Its action is based on the assumption that AGE are formed in a pathway involving reactive α-dicarbonyl intermediates. The AGE-breakers Nphenacylthiazolium bromide (PTB) or the chloride form developed by Alteon ALT-711, 3-phenacyl-4, 5-dimethylthiazolium chloride, is able to break this α-dicarbonyl bond, and can thereby remove established AGEcrosslinks (*Table 3*). However, taking into account the high reactivity of these α-dicarbonyl bonds, a crosslink containing this bond is not likely to occur. Because in vitro and in vivo studies indeed confirm the ability of PTB to actually diminish AGE derived crosslinks, therefore, future research should be focused on finding different mechanisms by which PTB breaks AGE crosslinks. Wolffenbuttel et al. performed the first in vivo study in rats, showing that chronic treatment with an AGE-breaker restored large artery properties in experimental diabetes [230]. In a study in aging dogs ALT-711 1mg/kg reversed the age-related increased stiffness of the myocardium within four weeks [231]. Left ventricular stiffness was reduced by approximately 40%. Studies on the effects of ALT-711 on older rhesus monkeys showed that an injection of 10 mg/kg ALT-711, every other day for three weeks, improved vascular compliance and elasticity [232]. The improved vascular compliance persisted over time, with maximum improvement seen six weeks after the end of ALT-711 treatment, and a gradual return to baseline 39 weeks after treatment was stopped.

After phase I clinical studies with ALT-711, Kass et al. described last year a randomized, double blind, placebo controlled trial in 93 individuals over the age of 50, with as selection criterion a systolic blood pressure > 140 mmHg and pulse pressure of

at least 60 mmHg [233]. Treatment consisted of ALT-711 210 mg daily or placebo for eight weeks, in addition to usual antihypertensive treatment. ALT-711 lowered pulse pressure with 5.6 mmHg drops in the treated group compared to a -0.5 mmHg drop in patients receiving placebo. ALT-711 significantly improved large artery compliance and distensibility by 11% to 18%, compared to placebo. The drug is now tested in several phase II trials in isolated systolic hypertension, addressing the vascular and left ventricular effects (Alteon website: SAPPHIRE and SILVER trials) and in diastolic heart failure.

AGE-RAGE Interaction Blockers

As discussed above, AGE-RAGE interactions may be important in numerous diseases. Attempts to block the deleterious effects of AGE at the receptor level are still in an experimental phase [74].

Prevention of other AGE-Induced effects

A novel approach in preventing deleterious effects of AGE has been proposed by Okamoto. He used incadronate to prevent AGE-induced angiogenesis from microvascular endothelial cells probably by blocking protein farnesylation [234].

Cerivastatin, a lipid lowering drug from the class of statins, prevents the AGE-induced increase in NF-kappaB and activator protein-1 activity, suppressed VEGF mRNA upregulation in cultured endothelial cells [235].

Interventions Aimed at Reducing Exogenous AGE, Food and Smoking

Vlassara addressed the effect of food derived proinflammatory AGE on inflammatory mediators in diabetic subjects by comparing otherwise comparable diets with a high AGE content or with a 5-fold lower AGE content. Serum AGE, measured as CML-sensitive ELISA, were approximately 70% higher on the high AGE compared to the low AGE diet, AGE-LDL was approximately 40% higher. The levels of the endothelial adhesion molecule VCAM-1and mononuclear tumor necrosis factor (TNF) alpha were considerably higher during the high AGE diet [236]. Although the study was too small

to draw definite conclusions, the results suggest that high AGE diets may result in higher levels of inflammatory mediators in diabetes patients and so contribute to the chronic complications. In apoE-deficient mice in whom a femoral artery injury had been induced, a low AGE diet compared to a diet with a tenfold higher AGE content resulted, 4 weeks after the injury in a decrease in neointima formation and a less stenotic luminal area [237]. Markedly lower number of macrophages and a reduced number of smooth muscle cells, associated with a 40% decrease in circulating AGE levels and AGE deposition in endothelial cells, smooth muscle cells and macrophages of the neointima in the low AGE diet mice were observed. The same group tested the effects of a low AGE diet also in two diabetic mouse models for type 1 and type 2 diabetes, respectively. The mice on a low AGE diet had a marked reduction in the diabetic nephropathy glomerular lesions and an extended survival [238]. In the renal cortex expression of transforming growth factor-beta1, laminin B1 mRNA and alpha-IV collagen mRNA was lower in the low AGE diet group, along with reduced serum and kidney AGE levels. The relation between diet and exogenous AGE load and resulting (serum) AGE levels is not always straightforward: Sebekova expected to find higher AGE levels in omnivores eating more heat-treated food would be higher than in vegetarians cooking their food. However, plasma AGE levels (measured with ELISA for CML, also plasma fluorescence) were higher in the vegetarians [239]. More specific food substances, for example green tea extracts, have been tested for their effect on protein glycation and oxidation in vitro [240]. Gugliucci found that in vitro extracts of the flavonoid-rich herbal species Achyrocline saturoides and Ilex paraguariensis inhibited the effects of the reactive carbonyl compound methylglyoxal on heparin activation of antithrombin III and plasminogen activity [241].

CONCLUSIONS

AGE have evolved from noxious bystanders of long-time glycation to endproducts of both oxidative and carbonyl stress. In association with the expansion in the biological effects of AGE, this makes AGE the primary targets for pharmaceutical interventions.

Pharmaceutical strategies can be aimed both at prevention of AGE formation and at AGE breaking. A weak point remains the dearth of feasible AGE assay methods available for clinical practice to monitor the effects of such pharmaceutical interventions. Introduction of reproducible, easily available assays of tissue AGE accumulation are warranted for a more focused application of treatment strategies.

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CHAPTER 4

Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes

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ABSTRACT

Introduction Advanced glycation end products (AGEs) are thought to have a role in the pathogenesis of diabetes complications. We recently reported the association between skin autofluorescence, as a measure of tissue AGE accumulation, and diabetic neuropathy in a selected diabetic population. In this study, we investigated the relation between skin autofluorescence and clinical variables including micro- and macrovascular complications in a type 2 diabetes primary care population.

Methods Clinical data and skin autofluorescence were obtained in the type 2 diabetes group (n=973) and in a control group (n=231). Skin autofluorescence was assessed by illumination of the lower arm with a fluorescent tube (peak intensity ~370 nm).

Results Skin autofluorescence was significantly higher in type 2 diabetic patients compared with control subjects in each age category. Multiple regression analysis showed significant correlation of skin autofluorescence with age, sex, diabetes duration, BMI, smoking, HbA1c, plasma creatinine, HDL cholesterol, and albumin-to-creatinine ratio in the type 2 diabetes group (R^2=25%) and with age and smoking in the control group (R^2=46%). Skin autofluorescence was significantly higher in the type 2 diabetes group, with both micro- and macrovascular disease, compared with the group without complications and the group with only microvascular complications.

Conclusion This study confirms in a large group of type 2 diabetic patients that skin autofluorescence is higher compared with age-matched control subjects and is associated with the severity of diabetes-related complications. Skin autofluorescence reflecting vascular damage might be a rapid and helpful tool in the diabetes outpatient clinic for identifying diabetic patients who are at risk for developing complications.

INTRODUCTION

The formation of advanced glycation end products (AGEs) is increased in diabetes [1–3]. Accumulated AGEs have deleterious effects on the vascular wall, contributing to the development of micro- and macrovascular disease, as shown especially in type 1 diabetes [4–8]. The Diabetes Control and Complications Trial substudy on skin

collagen glycation found strong associations between skin AGE levels and long-term diabetes complications in type 1 diabetes, which persisted after adjustment for HbA1c [7]. The relation between AGE accumulation and outcome has been studied very little in type 2 diabetes. Besides, several laboratory assessments of AGEs are used worldwide. In the earlier studies of the 1990s, AGEs were mostly assessed in tissue by collagen linked fluorescence (CLF) or specific AGE measurements (pentosidine, carboxymethyllysine) [4,9,10]. During the last few years, AGEs are increasingly measured in serum or plasma using high-performance liquid chromatography, enzyme-linked immunosorbent assay, or fluorescence. The blood compartment is more practical for repeated measurements than tissue requiring biopsies, but plasma AGE assays are unfortunately less reproducible and less well related to tissue contents of AGEs [11,12]. We recently described a tool to noninvasively assess tissue AGEs in vivo using skin autofluorescence [13]. This method utilizes the fluorescent properties of AGEs, like the extensively used CLF method, and has been validated with specific AGE measurements and CLF in skin biopsies [13]. By linking skin autofluorescence to AGE accumulation and, therefore, to cumulative glycemic and oxidative damage in diabetes, we aim to create a tool that is able to give rapid impression of the risk for diabetes complications. Using this tool in a prospective cohort of type 2 diabetic patients, we aim to address the predictive value of skin autofluorescence on diabetes-related complications. This study involves a cross-sectional analysis of the baseline data of this cohort to study the association between skin autofluorescence and clinical variables, including the presence of complications at baseline.

METHODS

Patients We recruited subjects participating in the ZODIAC (Zwolle Outpatient Diabetes project Integrating Available Care) study, which has previously been described [14]. In short, this study investigates the effects of a shared care project in a primary care population– based cohort of type 2 diabetic patients in the eastern part of the Netherlands. All patients of 32 general practitioners receiving their diabetes support

in the primary care setting and who additionally visited the diabetes outpatient clinic annually were approached. Patients were included from May 2001 to May 2002. Patients with cognitive disability or terminal diseases were excluded from the ZODIAC study and, consequently, also ineligible to the present study. After obtaining informed consent, autofluorescence was measured in 1121 of the 1450 patients (77%) that visited the outpatient clinic during the inclusion period. Additionally, skin autofluorescence was measured in a nondiabetic control group of 231 consecutive preoperative evaluation visitors of the outpatient clinic who did not have a history of diabetes, cardiovascular events, or renal disease [15]. The study was approved by the local ethical committee.

Autofluorescence Skin autofluorescence was assessed by the autofluorescence reader (AFR: patent PCT/NL99/00607, prototype of current AGE Reader; DiagnOptics BV, Groningen, the Netherlands) as described previously [13]. In short, the autofluorescence reader illuminates a skin surface of ~4 cm^2, guarded against surrounding light, with an excitation light source (8-W blacklight; Philips) between 300 and 420 nm (peak excitation ~370 nm). Emission light and reflected excitation light from the skin are measured with a spectrometer (AVS-USB2000; Avantes, Eerbeek, the Netherlands), in the 300 to 600 nm range, using a 50-µm glass fiber (Farnell, Leeds, U.K.). Measurements were performed at room temperature, while patients were in a seated position, at the volar side of the arm 10 cm below the elbow fold. Since skin pigmentation may influence autofluorescence by light absorption [16], autofluorescence was calculated by dividing the average emitted light intensity per nanometer in the range 420–600 nm by the average excitated light intensity per nanometer in the range 300–420 nm. Autofluorescence was expressed in arbitrary units (a.u.) and multiplied by 100. Skin reflection was calculated in the range 300–420 nm by dividing the mean intensity reflected from the skin by the mean intensity reflected from a white Teflon block (assuming 100% reflectance). Autofluorescence measurements were performed in 1121 type 2 diabetic patients at two different locations, with two identical autofluorescence reader systems, by six diabetes nurses. Measurements in 148 patients (13%) had to be discarded: 74 for improper previous

calibration, 36 showed light leakage from surrounding light, 7 because the tube was switched off inadvertently, and 31 because of too dark skin type, resulting in almost complete absorption of all the excitation light (skin reflection <7.5%), leaving too little light to measure autofluorescence with the current set up. Autofluorescence measurements in the control group were performed by one examiner. None of the measurements in the control group were discarded. Thus, autofluorescence measurements in 973 type 2 diabetic patients and 231 control subjects were available for analysis. Overall, the autofluorescence measurements and mean age-corrected autofluorescence per measuring month (p=0.3), per examiner (p=0.6), and per autofluorescence reader system (p= 0.1) did not differ significantly.

Clinical data Clinical data are derived from the Diabetic Electronic Monitoring System containing all data of the diabetic patients since the start of the shared-care project (1998) [17]. Clinical data and laboratory results obtained on the date of the autofluorescence measurement were used in the analysis. The blood pressure was a seated single measurement, obtained after 5 min rest, using an aneroid device. HbA1c was measured with a Primus CLC-385 using boronate affinity chromatography and high-performance liquid chromatography (reference value 4.0–6.0%). A Roche/Hitachi MODULAR analyzer was used for serum creatinine (kinetic colorimetric assay), nonfasting total cholesterol (cholesterol sterase/peroxidase enzymatic method), nonfasting HDL cholesterol (HDL-Cholesterol, homogeneous enzymatic colorimetric test), nonfasting triglycerides (lipase glycerol kinase enzymatic method), and urinary microalbumin (Tina-quant Albumin, immunoturbidimetric assay). Retinal photography was scored by an independent ophthalmologist as absent, background, or proliferative retinopathy. Microalbuminuria was defined as an albumin-to-creatinine ratio >3.5 in women or >2.5 in men, in two subsequent samples (at baseline and in the year before baseline) or once in the year before baseline while using an ACE inhibitor at baseline. Diminished sensibility at least at one foot was considered as neuropathy (tested with a 5.07 Semmes Weinstein monofilament, applied on three areas of each foot) [18]. The presence of microvascular complications was defined as meeting the criteria of

microalbuminuria, neuropathy, and/or at least background retinopathy. Cardiovascular disease was defined as ischemic heart disease (ICD-9 codes 410 – 414 and/or a history of coronary artery bypass surgery or percutaneous coronary intervention), cerebrovascular accidents, or peripheral vascular disease (surgical intervention, claudication, and/or diminished or absent pulsations of ankle or foot arteries). The presence of macrovascular complications is defined when meeting at least one aspect of cardiovascular disease. The following clinical data of the control group were collected: age, sex, current tobacco use, BMI, and blood pressure. An independent anaesthesiologist classified the subjects according to the American Society of Anaesthesiologists (ASA) classification, ASA class 1–5 (ASA 1 means a healthy patient without medical problems, and ASA 5 means moribund, not expected to live for another 24 h) [19]. The large majority of the control group were classified as ASA 1 and 2 (>90%).

Statistical analyses Multiple linear regression analysis was used to determine independent relations between skin autofluorescence and clinical variables. ANOVA was applied to compare differences between groups. Calculation of sample size of the diabetic population was based on a Cox regression power analysis. As the present cross-sectional study is part of a follow-up study in which cardiovascular end points will be the most critical end point, 741 patients are needed to detect a 6% difference in event-free surviving proportion (0.93 vs. 0.87) with 80% power and α=0.05 in a two-sided test, with 17% lost to follow-up (200 of 1200). Sample size of the nondiabetic control population was calculated according to formula of Altman [20]. In our previous study, skin autofluorescence was 20–30% higher in diabetic patients [13]. This was a heterogeneous group, including type 1 and type 2 diabetic patients. Furthermore, the mean HbA1c was higher (7.8%) than the mean HbA1c (7.1%) of the type 2 diabetic patients in this study (calculated in an interim analysis after inclusion of 475 patients). This taken into account, we considered a difference of 10–15% as clinically relevant. According to the nomogram of Altman [20], with 80% power and α=0.05, a sample size of 200 nondiabetic control subjects was needed.

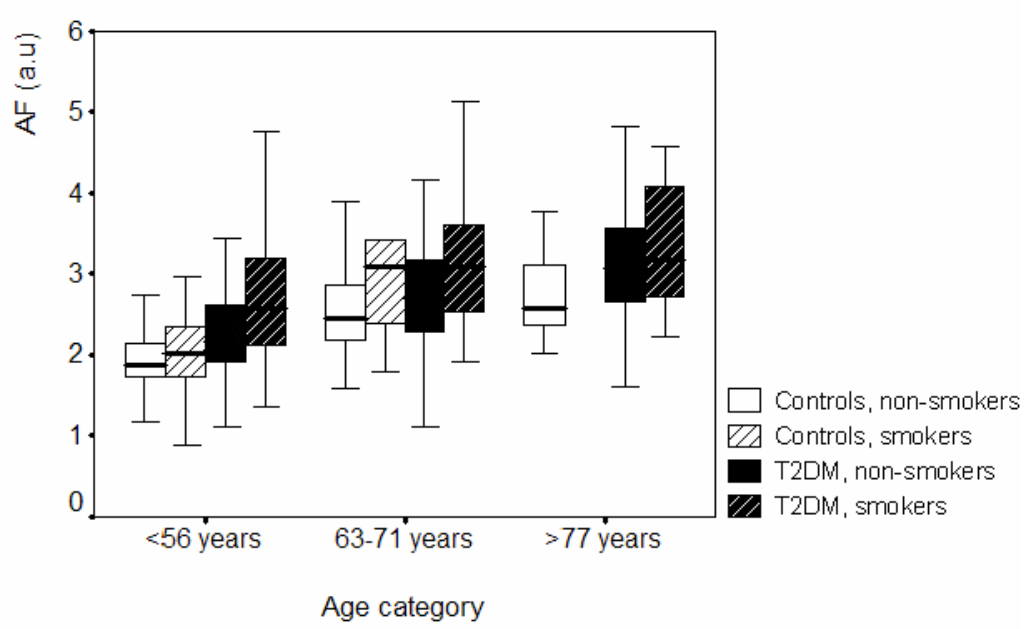
Table 1. Clinical Characteristics of the type 2 diabetic population and the control group. Mean skin autofluorescence of the total groups and per age-category are shown. Values are expressed as mean (SD) and % when indicated.

	T2DM (*n*=973)	**Controls** (*n*=231)
Characteristic		
Age (years)	66 (11)	52 (17)
Male gender	47%	38%
Caucasian	97%	100%
Smoking	19%	30%
Body mass index (kg/m2)	29 (5)	27 (5)
Systolic blood pressure (mmHg)	146 (20)	144 (22)
Diastolic blood pressure (mmHg)	81 (10)	82 (11)
Diabetes duration, (years)	*4.2 (1.6-8.3)	-
HbA1c (%)	7.0 (1.3)	#
Creatinine (µmol/l)	96 (20)	#
Creatinine clearance (Cockroft-formula) (ml/min)	76 (27)	#
Urinary albumin/creatinine ratio	*1.47 (0.8-4.1)	#
Total cholesterol (mmol/l)	5.2 (1.0)	#
HDL (mmol/l)	1.3 (0.3)	#
LDL (mmol/l)	2.9 (0.9)	#
Triglycerides (mmol/l)	2.3 (1.3)	#
Microvascular disease	49%	-
Macrovascular disease	39%	-
Autofluorescence:		
Skin autofluorescence (a.u), total group	2.79 (0.8)	2.14 (0.6)
40-49 years (*n*=64; *n*=44)	¶2.17 (0.5)	1.84 (0.4)
50-59 years (*n*=199; *n*=47)	¶2.57 (0.7)	2.12 (0.3)
60-69 years (*n*=278; *n*=32)	2.69 (0.7)	2.50 (0.6)
70-79 years (*n*=307; *n*=37)	¶3.04 (0.8)	2.76 (0.6)
>80 years (*n*=108; *n*=7)	3.17 (0.7)	2.89 (0.5)

*Median and Interquartile range. ¶p<0.01 (t-tests in horizontal direction, per age-category). #Reference values of the laboratory. HbA1c 4.0-6.0%, Creatinine 70-110 µmol/l, Creatinine clearance (Cockroft-formula) 80-120 ml/min, Urinary albumin/creatinine ratio 0-2.5, Total cholesterol 3.5-5.0 mmol/l, HDL 0.9-1.7 mmol/l, LDL 3.6-4.4 mmol/l, Triglycerides 0.6-2.2 mmol/l

RESULTS

In the analysis of this study, 973 type 2 diabetic patients and 231 control subjects were included. The medical characteristics are depicted in *Table 1*. All data were distributed normally, except for diabetes duration and albumin-to-creatinine ratio. Mean age in the control group was 14 years lower compared with the type 2 diabetes group, and there were more male subjects in the type 2 diabetes group (47% vs. 38% in the control group). In the type 2 diabetes group, median diabetes duration was 4.2 years (mean 6.3 years). Mean HbA1c was 7.0%, while 16% of the diabetic population was treated with insulin and 84% on noninsulin strategies (diet and/or oral agents). Microvascular complications were present in 49% of the patients (prevalence of retinopathy, 20%; microalbuminuria, 21%; and neuropathy, 28%). The prevalence of macrovascular disease was 39% (ischemic heart disease, 21%; cerebrovascular disease, 8%; and peripheral vascular disease, 22%). There were no patients with (near) end-stage renal disease. Skin autofluorescence. Overall, mean skin autofluorescence was 33% higher (2.79 a.u.) in the type 2 diabetes group compared with the control group (2.14 a.u.). In both type 2 diabetic and control subjects, autofluorescence was higher at each increment in age category ($p<0.01$), *Table 1*. Autofluorescence was also higher in the type 2 diabetes group compared with the control group at each specific age category, which was significant ($p<0.01$) in the age categories 40–49, 50–59, and 70–79 years, and nonsignificant ($p=0.1$, $p=0.3$) in the age categories 60 – 69 and >80 years. Sample sizes of the latter categories were very small in the control group. Multiple regression analysis showed that 25% (R^2) of the variance of skin autofluorescence could be predicted by age ($\beta=0.35$, $p<0.0001$), female sex ($\beta=0.17$, $p<0.0001$), current tobacco use ($\beta=0.14$, $p<0.0001$), diabetes duration ($\beta=0.10$, $p<0.01$), plasma creatinine ($\beta=0.15$, P 0.0001), HbA1c ($\beta=0.10$, $p<0.001$), albumin-to-creatinine ratio ($\beta=0.10$, $p<0.001$), BMI ($\beta=0.08$, $p<0.01$), and HDL cholesterol ($\beta=0.07$, $p<0.05$). Blood pressure and other nonfasting lipids were not significant in this model. In the nondiabetic control group, multiple regression analysis showed that 46% (R^2) of the variance of skin autofluorescence could be predicted by age ($\beta=0.71$, $p<0.001$) and current tobacco use ($\beta=0.17$, $p<0.001$).



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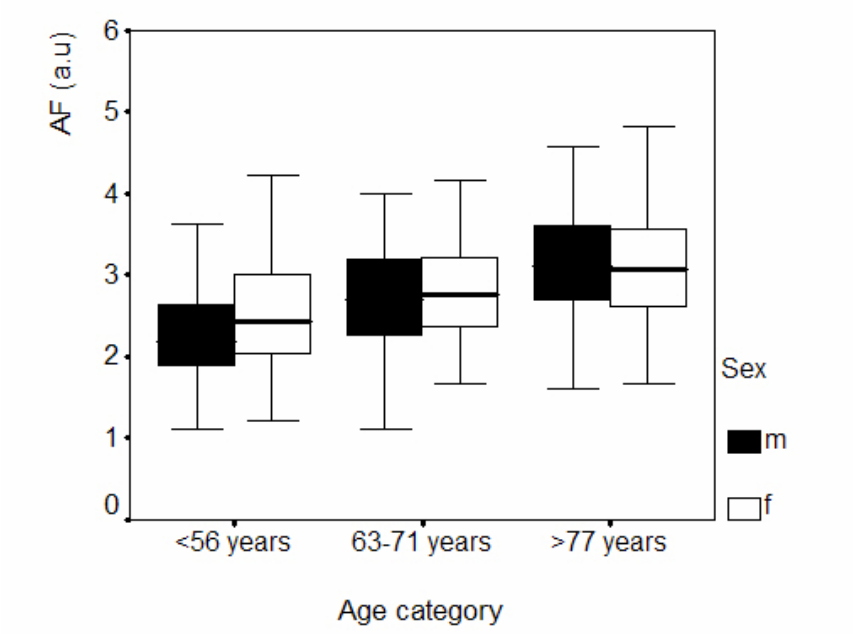

B

Figure 1. These boxplots show the distribution of skin autofluorescence (AF, y-axis) per quintile of age. Only the first, third and fifth age-quintile are shown (x-axis). *Panel A* shows the effect of smoking in control subjects compared to type 2 diabetes groups. The boxplot of the smoking controls in the age category above 77 years has not been depicted because of the too small group size. *Panel B* shows the differences in AF due to sex at different age-categories in the type 2 diabetes population. Skin AF is significantly higher in female patients under 56 years of age ($p<0.01$). The horizontal line within a box represents the median, the lower and upper end of a box are the first and third quartile respectively. The lower line and the upper line outside the box represent the 5th and 95th percentile, respectively.

In this multivariate analysis, the univariate correlation of autofluorescence with BMI ($r=0.2$, $p<0.05$) and systolic blood pressure ($r=0.3$, $p<0.001$) disappeared. Smoking was independently and positively related to autofluorescence in both groups. Smoking was more prevalent at younger age in both groups; in the type 2 diabetes group, the prevalence of smoking was 27% before age 65 years and 14% after age 65 years and in the control group, 37% and 13%, respectively. *Figure 1A* illustrates the effect of smoking on skin autofluorescence in control and type 2 diabetic subjects. Female sex was independently and positively associated with skin autofluorescence in type 2 diabetes. No sex relation with autofluorescence was found in the control group. Further analysis in the type 2 diabetes group showed that autofluorescence was significantly higher in women under age 56 years compared with men (2.56 vs. 2.30 a.u., respectively; $p<0.01$), *Figure 1B*. There was no significant sex-related difference of autofluorescence in the higher age categories. *Figure 2* shows the mean skin autofluorescence in the type 2 diabetic population per complication category with the 95% CI. Mean skin autofluorescence was significantly higher in the group with both micro- and macrovascular disease compared with the group without complications and the group with only microvascular complications (mean 3.12 [95% CI 3.01–3.23] vs. 2.57 [95%CI 2.50–2.65] and 2.71a.u. [95%CI 2.62–2.80]; $p<0.001$). Autofluorescence in the group with only macrovascular complications (2.91 a.u [95%CI 2.78 –3.03]) was higher than in the group without complications. These differences remained significant when autofluorescence was corrected for age. The same result was found by categorizing the type 2 diabetes group with both micro- and macrovascular disease and the type 2 diabetes group without complications in age decades. In each age decade >60 years, skin autofluorescence was significantly higher in type 2 diabetic patients with complications versus type 2 diabetic patients without complications (40-49 years: 2.27 vs. 2.08 a.u. [p=NS]; 50-59 years: 2.77 vs. 2.55 a.u. [p= NS]; 60-69 years: 2.83 vs. 2.53 a.u.; 70-79 years: 3.24 vs. 2.86 a.u; and >80 years: 3.47 vs. 3.01 a.u. [$p<0.05$]).

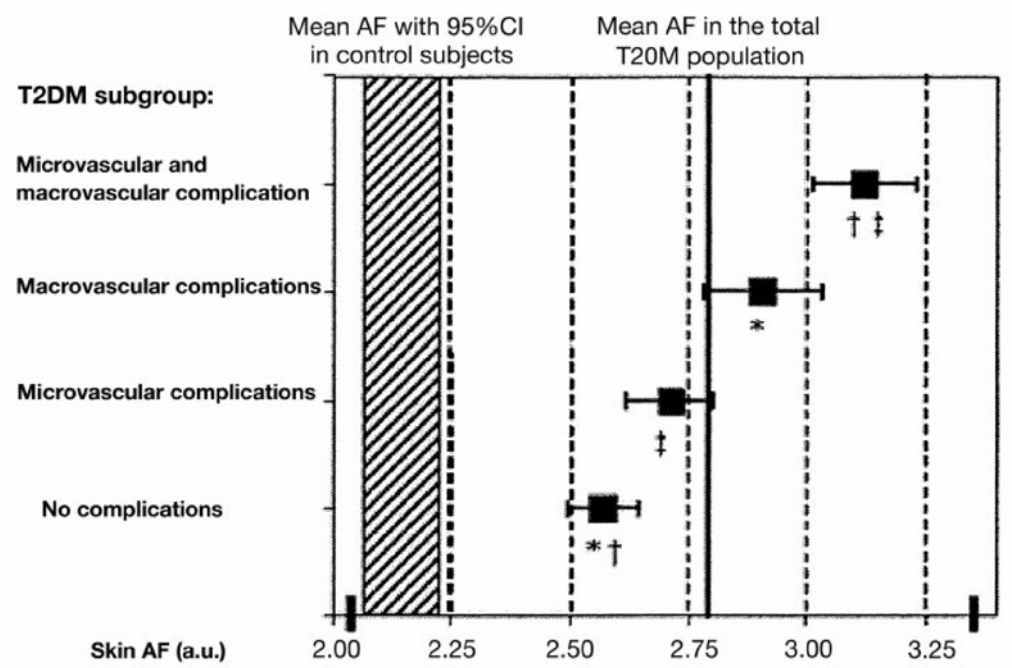

Figure 2. Mean Skin AF with 95% Confidence interval in different categories of complications in the type 2 diabetes population. Skin AF is represented at the X-axis and the complication categories are depicted at the Y-axis. Matching symbols means significant difference between the groups ($p<0.001$). These differences remained significant after age-correction of skin AF. The lined column represents the mean skin AF with 95% confidence interval of the control group.

DISCUSSION

This study confirms that in a large group of type 2 diabetes patients, skin autofluorescence higher compared with age-matched control subjects. Moreover, skin autofluorescence was associated with a graded increase in the presence and severity of diabetes-related complications.

Known diabetes duration was relatively short, and, moreover, metabolic control was good (mean HbA1c 7.0%) compared with the previously reported smaller group with increased skin autofluorescence [13]. Thus, our type 2 diabetes group might be expected to represent a more or less “healthy” diabetes population relatively unaffected

by chronic complications. However, about half of this group was already diagnosed with micro- or macrovascular complications.

HbA1c had a small independent contribution to autofluorescence in our study. Sharp et al. [21] found no correlation with HbA1c and serum low–molecular weight AGEs (LMW-AGEs). They suggest that glycaemia itself may not be responsible for increased AGE accumulation in diabetes, but other factors are involved. However, it can also be hypothesized that the restricted relation between glycaemia (HbA1c) and AGEs is caused by the short turnover time of HbA1c. HbA1c represents metabolic control of the last ~8 weeks, which implies a rather "short-term memory" of glycemic stress. AGEs can also link to haemoglobin (Hb-AGE). Hb-AGE was slightly superior to HbA1c as a measure for metabolic control, which was attributed to the irreversible nature of AGEs [22]. However, the relatively short turnover time of haemoglobin remains the limiting factor for the use of Hb-AGE or HbA1c as a real long-term glycemic index. An earlier study that confirmed the importance of protein turnover in AGE accumulation estimated the half-life of skin collagen on 15 years [23]. Thus, skin AGE levels might provide a more "long-term memory" of glycemic stress and, therefore, be better in predicting complications. The Diabetes Control and Complications Trial substudy already demonstrated in type 1 diabetes that skin collagen glycation was a better predictor for diabetes complications compared with HbA1c [7,24].

Sex was an independent variable of skin autofluorescence in the type 2 diabetes group, with a higher autofluorescence in women (aged <56 years). This suggests an estrogen-related effect. The loss of sex difference in autofluorescence at an older age may be due to a decrease in skin collagen content in postmenopausal women [25]. Sex hormones influence the collagen turnover rate and is important in the rate of AGE accumulation. Another large study in type 2 diabetes found a similar independent positive relation between female sex and levels of LMW-AGEs [26].

BMI and HDL cholesterol also contributed significantly to autofluorescence in the regression model. Although the coefficients were low, these relations can be valid. It is possible that skin autofluorescence is affected by the skin content of lipoxidation

products, as some of these products are fluorescent [27]. These advanced lipoxidation end products are formed analogous to AGEs from reactive carbonyl intermediates through oxidation of polyunsaturated fatty acids instead of sugars. If an increase in subcutaneous fat mass and a decrease in HDL cholesterol are associated with increased formation of advanced lipoxidation end products, this might explain the contribution to skin autofluorescence.

This study further supports the earlier finding that smoking results in increased AGE accumulation, as we showed that smoking independently increases autofluorescence in both type 2 diabetic and control subjects in the multivariate analysis [28].

Skin autofluorescence had an explained variance, with the tested clinical variables in the regression analysis of 46% in the control group and 25% in the type 2 diabetes group. It should be noted that the total variance (SD^2) was approximately two times higher in type 2 diabetes, which implies that the explained variances in the control group and the type 2 diabetes group are about the same [$SD^2(1-R^2)$]. Sharp et al. [21], who measured fluorescent LMW-AGEs in plasma, also found an R^2 value of 25% and increased total variance in type 2 diabetic compared with control subjects.

One of the reasons for a higher total variance in the diabetic population might be that other "disturbing" disease-related factors exist. For example, interindividual differences in actual hyperglycaemic induced oxidative stress during the autofluorescence measurement [2]. It is also possible that genetic factors are involved in the individual susceptibility to glycation, as has been described previously [29].

There are some restrictions of the skin autofluorescence technique. First, non-fluorescent AGEs are not measured with our tool. In our previous study, however, skin autofluorescence was well correlated with levels of both fluorescent (pentosidine) and nonfluorescent AGEs (e.g., carboxymethyllysine) assessed in skin biopsies, which suggests a parallel trend formation of AGEs. It is unclear, so far, whether skin autofluorescence represents some intracellular-produced AGEs, which may be nonfluorescent. Secondly, other fluorophores might be measured (e.g., NADH, which has an overlapping excitation spectrum: 350–370 nm) [16]. Finally, the large majority of our study population was Caucasian. Some non-Caucasian type 2 diabetes patients

had to be excluded because of their skin type. The tool is in further development to measure autofluorescence in dark skin. Skin autofluorescence has recently shown clinical relevance as a strong and independent predictor of total and cardiovascular mortality in a study in patients with end-stage renal disease undergoing hemodialysis [30].

In this study, we showed that increased levels of skin autofluorescence was related to the extent of diabetes related complications. In a 4-year follow-up study, the progression of microvascular and macrovascular complications, as well as mortality, is now evaluated in the current study group of type 2 diabetes patients to analyze whether skin autofluorescence contributes to the prediction of the development or progression of diabetes complications. If this hypothesis is confirmed, skin autofluorescence, reflecting vascular damage, will become an easy tool for clinical practice to identify diabetic patients who are at increased risk for developing vascular complications.

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CHAPTER 5

Skin autofluorescence provides additional information to the UKPDS risk score on estimating cardiovascular prognosis in type 2 diabetes mellitus

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ABSTRACT

Introduction The United Kingdom Prospective Diabetes Study (UKPDS) risk-engine has become a standard for cardiovascular (CV) risk-assessment in type 2 diabetes mellitus. Skin autofluorescence (AF) was recently introduced as an alternative tool for cardiovascular risk-assessment in diabetes. We investigated the prognostic value of skin autofluorescence for cardiovascular events, in combination with the UKPDS risk score in a type 2 diabetes primary care cohort.

Methods Clinical data, UKPDS risk score and skin autofluorescence were obtained at baseline in 2001-2002 in the type 2 diabetes group (n=973). Follow-up data concerning fatal and non-fatal CV-events (=primary endpoint) were obtained until 2005. Patients were classified as 'low risk' when their 10-yr UKPDS risk score for fatal CV-events were <10%, and 'high risk' if >10%. Skin autofluorescence was measured noninvasively with an autofluorescence reader. Skin AF was classified by the median (<median as low risk, >median as high risk).

Results Incidence of CV-events was 119 (44 fatal, 75 non-fatal). In multivariate analysis skin autofluorescence, age, sex and diabetes duration were predictors for the primary endpoint. Addition of skin autofluorescence to the UKPDS risk engine resulted in reclassification of 55 of 203 patients from the low risk to the high risk group. The 10-yr CV-event rate was higher in patients with a UKPDS score >10% when skin AF was above the median (55.8% vs. 38.9%)

Conclusion Skin autofluorescence provides additional information to the UKPDS risk engine which can result in risk-reclassification of a substantial number of patients. It furthermore identifies patients who have a particularly high risk for developing cardiovascular events.

INTRODUCTION

Increased formation and accumulation of advanced glycation endproducts (AGEs) is one of the pathogenetic mechanisms of accelerated atherosclerosis in type 2 diabetes [1]. Both cross-linking of proteins by AGEs, and receptor-mediated cellular activation

contribute to loss of vascular elasticity and to propagation and maintaining of inflammation, contributing to the development of microvascular and macrovascular disease. The DCCT-EDIC substudy on skin collagen glycation, found that skin tissue AGE levels predict long-term diabetic complications in type 1 diabetes, also after adjustment for HbA1c [2,3].

Recently, skin autofluorescence (AF) has emerged as a noninvasive and reproducible tool to estimate the skin tissue AGE level. Skin AF has been validated against skin levels of several specific AGEs, and against a classical assay method for AGEs, collagen-linked fluorescence, in different populations (diabetes, renal failure and controls) [4-6]. Skin AF was consistently shown to be related to both micro- and macrovascular complications in type 2 diabetes, and to be a strong and independent predictor of cardiovascular mortality in patients with diabetes mellitus, and in haemodialysis patients [5,7,8].

For a cardiovascular risk biomarker like skin AF to be useful in clinical practice, it is mandatory that it adds information to conventional risk factors or risk models in unselected cohorts. Several simulation models have been developed over the last few years to estimate the risk for future occurrence of diabetes-related complications. The UKPDS risk-engine has emerged as the most used tool to predict complications in type 2 diabetes [9]. Although it was developed in newly diagnosed type 2 diabetes, it is now also used in patients with known diabetes. Still the incorporation of established cardiovascular risk factors, and the derived algorithms like the UKPDS risk-engine do not fully explain cardiovascular risk [10]. Combining the UKPDS risk score and AF might improve the prediction of cardiovascular complications, especially since AGE accumulation may reflect a distinct biological pathway in atherogenesis, not or only partly covered by other risk factors. Therefore, our aim was to assess the additional value of skin AF on top of that obtained from the UKPDS risk-engine for the prediction of cardiovascular morbidity and mortality, in primary-care well-controlled patients with relatively recently diagnosed type 2 diabetes.

METHODS

Patients The previously described cohort of 973 patients was used for this follow-up study [7]. In short, the cohort was recruited from subjects participating in the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC)-study, which investigated the effects of a shared-care project in a primary care population based cohort of type 2 diabetes patients in the eastern part of the Netherlands, since 1998 [11]. All known type 2 diabetes patients of 32 general practitioners who received their diabetes support in the primary care setting and additionally visited the diabetes outpatient clinic annually, were approached for participation. Patients were included from May 2001 until May 2002. The large majority was of Caucasian ethnicity (97%). Patients with cognitive disability or terminal diseases were not included in the ZODIAC-study and, consequently, also ineligible for the present study. This study was approved by the local ethical committee. Written informed consent was obtained from the participants.

Material and technique. Skin autofluorescence was assessed at baseline using the Autofluorescence Reader (a prototype of the current AGE Reader, DiagnOptics BV, Groningen, The Netherlands) as described previously [7]. In short, the Autofluorescence Reader illuminates a skin surface of ~4 cm2, guarded against surrounding light, with an excitation light source with peak intensity at ~370 nm. Emission light and reflected excitation light from the skin are measured with a spectrometer in the 300-600 nm range, using a glass fibre. Measurements were performed at the volar side of the arm. AF was calculated by dividing the average emitted light intensity per nm in the range between 420-600 nm by the average excitated light intensity per nm in the range between 300-420 nm. AF was expressed as arbitrary units (a.u.), and multiplied by hundred. Over all AF measurements, the mean age-corrected AF per measuring month, per examiner, and per AFR-system did not differ significantly. Repeated AFR measurements taken over a single day in control subjects and diabetic patients showed an overall Altman error percentage of 5.03%. Intra-individual seasonal variance among control subjects and diabetic patients showed an Altman error percentage of 5.87%.

Clinical data The methods of baseline clinical data collection and laboratory assessments have been described in detail elsewhere, including the definition of a history of any microvascular disease (retinopathy, neuropathy and/or nephropathy) or any macrovascular disease (coronary heart, cerebrovascular and/or peripheral vascular disease) [7]. Follow-up data, obtained in the period between the date of the baseline autofluorescence measurement and January 2005, were used in the analysis.

UKPDS. The UKPDS risk-score (10-year non-fatal CHD risk, 10-year fatal CHD/Stroke risk) was calculated with the UKPDS risk-engine (version 2.0, http://www.dtu.ox.ac.uk) using the values of the required variables collected at baseline (single observations).

Events. Fatal or non-fatal events were registered from the date of inclusion until January 2005. Fatal events were categorized as: due to coronary heart disease (CHD), International Classification of diseases (ICD-9) codes 410-414, cerebrovascular disease 430-438, other cardiovascular disease 390-409, 415-429, 439-459, sudden death 798-798.9, and non-cardiovascular deaths (all other causes). The coded causes of death were combined to total mortality (all codes) and cardiovascular mortality (390-459 + sudden death). The following non-fatal cardiovascular events were registered: CHD (myocardial infarction, coronary artery bypass surgery and percutaneous coronary intervention), major cerebrovascular accidents or peripheral vascular disease (amputation, percutaneous transluminal angioplasty, bypass surgery). In case of multiple non-fatal cardiovascular events during the follow-up period, only the first event was considered in the study. All cases and timepoints of cardiovascular death or events were adjudicated by two independent physicians unaware of the skin AF results, using the hospital and general practitioner records. The primary endpoint was 'any cardiovascular event' (fatal + non-fatal CV-events), the secondary endpoints were all-cause mortality + non-fatal CV-events, and all-cause mortality only.

Statistical analysis. The size of the cohort was calculated before the start of the study in 2001, allowing sufficient CV-events during the follow-up period to detect a 6% difference in event-free surviving proportion (93% vs. 87%) [7,12]. Incidence rates of

endpoints were calculated per 1000 person-years. Univariate and multivariate Cox regression analysis were performed for the primary endpoint and the secondary endpoints. For the analysis of the possible additional information of skin AF to the UKPDS risk score, the participants were divided in those with a 10-years UKPDS risk for a fatal CV-event above and below 10%, and in those with a skin AF value above and below the median, resulting in four groups. The cut-off point of the UKPDS risk score was chosen because it is used in the Dutch cardiovascular risk management guidelines 2007 as a treatment decision cut-off value [13]. The classification of skin AF above and below the median was determined previously as a result of receiver-operating curve (ROC) analyses (non published data): ROC curves of skin AF expressed as continuous variable, or using classification defined by median, tertile and quartile were constructed for the endpoint all cause mortality. Area under the receiver-operating curve (AUC) using median-skin AF was highest: 0.63, 95% CI 0.58-0.69. AUC using AF on a continuous scale was 0.61, 95% CI 0.55-0.67, AUC using AF>2nd tertile 0.59 (95%CI 0.52-0.65), or AUC using AF in highest quartile 0.57 (95%CI 0.51-0.64) . Therefore, when we set up the methods for this manuscript, we decided to use median skin AF in the analysis. For the analysis of the possible additional information of skin AF to the UKPDS risk score we performed reclassification analyses, calculations of AUCs of 2 models with and without skin AF [14,15], and construction of Kaplan-Meier curves with the cumulative incidence of events in the four subgroups. Differences between the Kaplan-Meier curves were tested using Log rank test.

$P<0.05$ (two-tailed) was considered significant. Increment of AUC of 0.025 was considered clinically relevant. This study was not powered to reach statistical significance at an AUC increase of 0.025, which needs a sample size of >10.000 patients (80% power, $\alpha=0.05$).

Data are shown as mean ± SD, unless otherwise indicated.

Table 1. Baseline characteristics of the 967 T2DM-patients. Values are expressed as mean (SD) and % when indicated

Characteristic	***Mean (SD)***
Age (years)	66 (11)
Male gender	47%
Smoking	19%
Body mass index (kg/m2)	29 (5)
Systolic blood pressure (mmHg)	146 (20)
Diastolic blood pressure (mmHg)	81 (10)
Diabetes duration, (years)	[a]4.2 (1.6-8.3)
HbA1c (%)	7.0 (1.3)
Creatinine (µmol/l)	96 (20)
Creatinine clearance (Cockroft-formula)	76 (27)
Urinary albumin/creatinine ratio	[a]1.47 (0.8-4.1)
Total cholesterol (mmol/l)	5.2 (1.0)
Chol/HDL-ratio	4.3 (1.2)
HDL (mmol/l)	1.3 (0.3)
LDL (mmol/l)	2.9 (0.9)
Triglycerides (mmol/l)	2.3 (1.3)
Microvascular disease	53%
Microalbuminuria	25%
Retinopathy	20%
Neuropathy	29%
Macrovascular disease	39%
Coronary heart disease (CHD)	21%
Cerebrovascular disease	8%
Peripheral vascular disease (PVD)	22%
Skin autofluorescence (a.u.), mean	2.79 (0.8)
Skin autofluorescence (a.u.), median	[a]2.69 (2.26-3.19)
UKPDS risk score (%)	[a]27.4 (16.6-46.9)

[a]Median and Interquartile range

RESULTS

Group characteristics In 2001-2002, 973 type 2 diabetes patients were included. Six patients were lost to follow-up. Baseline characteristics of the 967 patients are depicted in *Table 1*. Mean age was 66 yrs, mean HbA1c 7.0% and mean diabetes duration was

6.3 yrs. Median follow-up was 1131 days (IQR 1019-1212 days). Forty-two patients died of non-cardiovascular causes. There were 119 cardiovascular events: 44 cardiovascular deaths, and 75 non-fatal cardiovascular events, corresponding with an incidence rate of this primary endpoint of 40.1 per 1000 person-years. A history of CHD was present in 204 patients. In these patients, the incidence of the primary endpoint was 75 per 1000 person-years at follow-up compared to 32 per 1000 person-years in patients without a history of CHD.

Validation of UKPDS risk engine in this cohort For all participants (including those with a history of CHD), the calculated median UKPDS risk score in 10 years for the primary endpoint was 35%. The calculated median UKPDS risk score in 3.2 years was 12.3%. This expected CV-event rate was not significantly different from observed: 13%, in this study.

Predictors in univariate and multivariate models of the primary and secondary endpoints Table 2 shows the univariate HR's for the different endpoints. The first multivariate model including all items of the UKPDS risk engine (except atrial fibrillation and ethnicity) plus AF, showed significant HR's for age, diabetes duration, female gender, and skin AF for the primary endpoint, see *Table 2*. Smoking, HbA1c, systolic blood pressure and lipid profile were not significant. The second multivariate model consisted of a history of microvascular disease, a history of a macrovascular disease, the 10-years UKPDS risk score for total fatal cardiovascular disease above 10%, and AF, and showed significant HR's for UKPDS risk score and for a history of macrovascular disease on all endpoints. Significant HR's were only observed for AF at both secondary endpoints. All HR's are presented in the lower part of *Table 2*. History of microvascular complications was not significant for any endpoint.

Table 2. Cox-regression analysis, univariate and multivariate

	Primary endpoint		1st Sec. endpoint		2nd Sec. endpoint	
Univariate	*HR*	*95% CI*	*HR*	*95% CI*	*HR*	*95% CI*
Age[a]	1.38[c]	1.18-1.58	1.46[c]	1.29-1.64	1.78[c]	1.51-2.05
Female gender	0.64[c]	0.45-0.92	0.69[c]	0.51-0.95	0.68	0.44-1.03
Diabetes duration[a]	1.22[c]	1.07-1.37	1.21[c]	1.08-1.34	1.22[c]	1.05-1.40
Current smoking	1.16	0.75-1.79	1.20	0.83-1.73	1.52	0.94-2.45
BMI[a]	0.94	0.76-1.12	0.94	0.78-1.09	1.01	0.80-1.22
Hypertension	0.96	0.64-1.43	1.06	0.74-1.52	1.24	0.75-2.07
Systolic blood pressure[a]	1.08	0.90-1.26	1.10	0.95-1.25	1.17	0.97-1.38
Diastolic blood pressure[a]	0.83	0.65-1.01	0.80	0.64-0.95	0.84	0.64-1.06
HbA1c[a]	1.04	0.87-1.23	1.03	0.89-1.20	1.03	0.83-1.26
Creatinine[a]	1.26[c]	1.12-1.40	1.26[c]	1.14-1.38	1.30[c]	1.15-1.45
Total Cholesterol[a]	0.96	0.80-1.15	0.87	0.74-1.02	0.80	0.64-1.00
Chol/HDL-ratio[a]	1.17	0.99-1.37	1.13	0.97-1.30	1.04	0.84-1.28
HDL-cholesterol[a]	0.86	0.78-1.02	0.85[c]	0.78-0.97	0.89	0.78-1.10
LDL-cholesterol[a]	0.97	0.81-1.16	0.94	0.80-1.10	0.95	0.77-1.82
Skin AF above median	1.80[c]	1.24-2.62	2.05[c]	1.48-2.84	3.13[c]	1.93-5.08
History of PVD	3.19[c]	2.22-4.58	3.01[c]	2.21-4.12	3.96[c]	2.59-6.06
History of CHD	2.48[c]	1.71-3.60	2.18[c]	1.57-3.01	1.98[c]	1.26-3.10
Albuminuria	1.76[c]	1.21-2.56	1.85[c]	1.35-2.55	2.50[c]	1.63-3.82
Neuropathy	1.49[c]	1.03-2.16	1.51[c]	1.09-2.07	1.85[c]	1.20-2.84
Retinopathy	1.23	0.81-1.88	1.04	0.71-1.53	1.17	0.70-1.95
Microvascular complications	1.25	0.87-1.81	1.33	0.97-1.83	2.04[c]	1.29-3.24
Macrovascular complications	4.33[c]	2.91-6.45	3.26[c]	2.36-4.52	2.99[c]	1.93-4.63
10-yrs CHD-risk[a,b]	2.19[c]	1.48-3.59	2.32[c]	1.63-3.56	3.55[c]	2.04-6.84
10-yrs risk for fatal CV-event[a,b]	1.80[c]	1.34-2.60	1.90[c]	1.46-2.61	2.62[c]	1.75-4.25
10-yrs risk for fatal CV-event <10%[a,b]	3.97[c]	1.94-8.13	3.57[c]	1.98-6.42	4.71[c]	1.91-11.63
Multivariate						
model 1	*HR*	*95% CI*	*HR*	*95% CI*	*HR*	*95% CI*
Age[a]	1.33[c]	1.11-1.56	1.41[c]	1.21-1.61	1.71[c]	1.40-2.02
Diabetes duration[a]	1.18[c]	1.01-1.35	1.15[c]	1.01-1.29	1.07	0.87-1.27
Female gender	0.59[c]	0.40-0.86	0.65[c]	0.46-0.90	0.59[c]	0.37-0.94
Smoking	1.18	0.75-1.86	1.24	0.84-1.82	1.71[c]	1.03-2.82
HbA1c[a]	0.97	0.79-1.18	0.98	0.82-1.16	1.00	0.78-1.26
Systolic blood pressure[a]	1.01	0.83-1.20	1.02	0.87-1.18	1.10	0.85-1.36
Total Cholesterol[a]	1.06	0.88-1.28	0.96	0.82-1.14	0.88	0.70-1.10
HDL Cholesterol[a]	0.86	0.77-1.04	0.86	0.78-1.00	0.94	0.80-1.24
Skin AF	1.28[c]	1.01-1.62	1.57[c]	1.10-2.25	2.05[c]	1.22-3.45
model 2						
Microvascular complications	0.86	0.59-1.26	0.94	0.68-1.30	1.40	0.88-2.25
Macrovascular complications	3.79[c]	2.51-5.72	2.74[c]	1.96-3.83	2.16[c]	1.38-3.40
10-yrs risk for fatal CV-event >10%[a]	2.79[c]	1.34-5.84	2.50[c]	1.36-4.58	2.78[c]	1.10-7.03
Skin AF	1.21	0.82-1.79	1.46[c]	1.04-2.05	2.18[c]	1.32-3.59

Primary endpoint: fatal + non-fatal CV-events, 1st secondary endpoint: non-fatal CV-events + all cause mortality, 2nd secondary endpoint: all cause mortality. [a]HR for continuous variables are expressed per SD increase. [b]calculated with the UKPDS Risk Engine. [c]significant HR.

Additional value of skin AF to UKPDS risk score for the primary and secondary endpoints. Reclassification of risk groups Table 3 shows multivariate Cox-regression analyses and comparison of AUCs for models with the UKPDS risk score (cut-off point 10%), with or without addition of skin AF (cut-off point median), for all three endpoints. In model 1, for the primary endpoint, the AUC of the model including skin AF increased from 0.581 to 0.618, which is relevant (>0.025), but not significant. For the 2nd secondary endpoint, the AUC increased significantly to 0.667 (p=0.03). AUC was highest in model 2 (AUC 0.718), including also microvascular, macrovascular complications and skin AF. The AUC of this model without skin AF was 0.712, so in model 2 AF had no relevant contribution in the prediction of the primary endpoint.

Table 4 presents the proportion of patients initially classified as having a 10-year risk of less than 10% or higher than 10%, who would be reclassified to a higher or lower risk category by having a skin AF lower or higher than the median. It appeared that 55 of 203 persons (27%) with a calculated 10-year fatal CV risk of <10% with the UKPDS risk-engine had an AF above the median, resulting in reclassification from low to high risk. The calculated 10-year CV-event rate (based on the observed CV-events in the first 3.2 years) in this group with low risk concerning the UKPDS risk score and high risk concerning skin AF, is 17.8%, which is 59% higher than the patients with low UKPDS risk score and skin AF below the median (11.2%). This difference was not significant (p=0.5). Within the high risk group (UKPDS risk score >10%), the calculated 10-year CV-event rate was significantly higher (55.8%) in patients with skin AF > median, compared to patients with skin AF <median (38.9%), p=0.049.

Figure 1 depicts this result, showing Kaplan-Meier survival curves for the primary end point of the four groups. For this endpoint, survival was significantly different between group 4 and all other groups (1: p<0.001, 2:p<0.05, 3: p<0.05), and between group 3 and group 1 (p<0.01). Similar differences between groups were found in the analysis concerning the secondary endpoints (survival curves not shown). When subjects with a known history of CHD at baseline were excluded from the analysis, similar differences between the same groups were also found for the primary end point, except that the difference between groups 3 and 4 lost significance (p = 0.053).

Table 3. Multivariate Cox-regression analyses and comparison of Areas under Receiver-Operating curve for models with the UKPDS risk score above 10%, with or without addition of skin autofluorescence to the models.

	Primary endpoint				1st secondary endpoint				2nd secondary endpoint			
Model 1	*HR*	*95% CI*	*AUC*	*p*[b]	*HR*	*95% CI*	*AUC*	*p*	*HR*	*95% CI*	*AUC*	*p*
10-yrs risk for fatal CV-event >10%[a]	3.97	1.94-8.13	0.581		3.57	1.98-6.42	0.581		4.71	1.91-11.6	0.583	
10-yrs risk for fatal CV-event >10%[a]	3.51	1.70-7.25			3.03	1.67-5.49			3.56	1.43-8.86		
Skin AF > median	1.52	1.05-2.22	0.618	0.29	1.75	1.26-2.44	0.635	0.08	2.64	1.62-4.31	0.667	0.03
Model 2												
Microvasc compl	0.88	0.61-1.28			0.98	0.71-1.36			1.53	0.96-2.44		
Macrovasc compl	3.92	2.61-5.87			2.91	2.09-4.06			2.45	1.57-3.83		
10-yrs risk for fatal CV-event >10%[a]	2.95	1.42-6.12	0.712		2.77	1.52-5.04	0.682		3.41	1.36-8.52	0.689	
Microvasc compl	0.86	0.59-1.26			0.94	0.68-1.30			1.40	0.88-2.25		
Macrovasc compl	3.79	2.51-5.72			2.74	1.96-3.83			2.16	1.38-3.40		
10-yrs risk for fatal CV-event >10%[a]	2.79	1.34-5.84			2.50	1.36-8.52			2.78	1.10-7.03		
Skin AF > median	1.21	0.82-1.79	0.718	0.86	1.46	1.04-2.05	0.705	0.46	2.18	1.32-3.59	0.715	0.50

[a]calculated with the UKPDS Risk Engine. [b]test between AUCs of model 1 and 2 with and without skin AF. CV = cardiovascular AF = autofluorescence. AUC = Areas under Receiver-Operating curve. Primary endpoint = fatal + non-fatal cardiovascular events; 1st secondary endpoint is all-cause mortality + non-fatal cardiovascular events; 2nd secondary endpoint = all-cause-mortality

Table 4. Risk reclassification for the primary endpoint (fatal + non-fatal cardiovascular events) in 967 type 2 diabetes patients by addition of skin autofluorescence to the UKPDS Risk Engine.

10-Year risk categories using the UKPDS Risk Engine		Total	[c]AF<median	AF>median	*p*	No (%) reclassified
<10%						
	n	203	148	55		55/203= 27%
	events[a]	8 (3.9%)	5 (3.4%)	3 (5.4%)		
	10-years event rate [b]	12.8%	11.2%	17.8%	0.500	
>10%						
	n	764	334	430		
	events	111(14.5%)	39(11.7%)	72(16.7%)		
	10-years event rate	47.90%	38.90%	55.80%	0.049	
All		967				
	events	119 (12.3%)				
	10-years event rate	40.5%				

[a]observed events during follow-up (~3-years). [b]calculation based on the observed incidence in the first ~3 years. [c]AF = skin autofluorescence

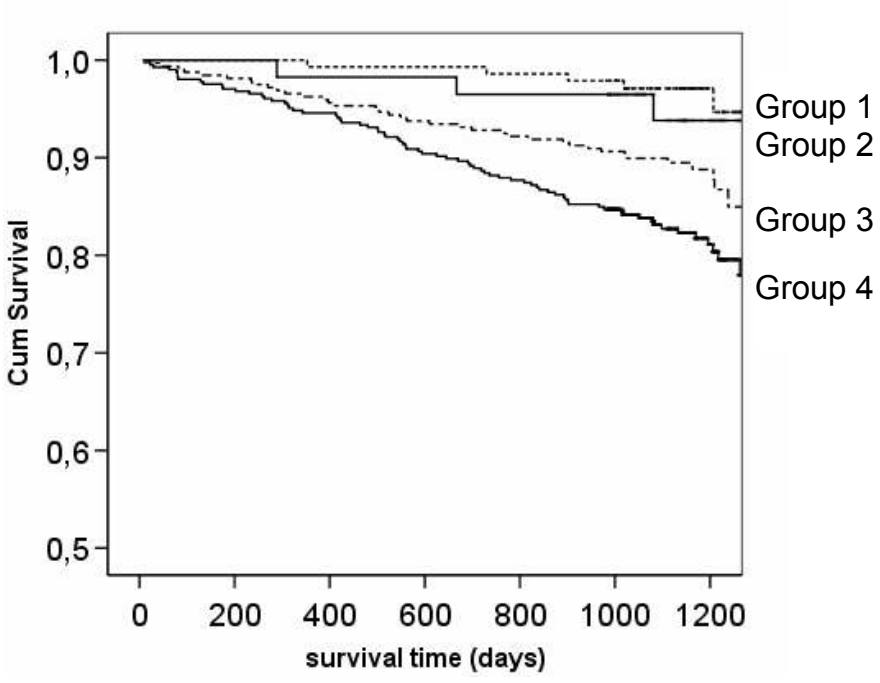

Figure 1. Kaplan-Meier survival curves for the primary endpoint for the four groups: Group1. AF < median and UKPDS risk score <10% (*n*=144). Group 2. AF > median and UKPDS risk score <10% (*n*=59). Group 3. UKPDS risk score >10% and AF < median (*n*=328). Group 4. AF > median and UKPDS risk score >10% (*n*=436). Survival was significantly different between group 4 and al other groups, and between group 3 and group 1.

DISCUSSION

This study shows that skin autofluorescence is of additional clinical value in the evaluation of risk for fatal and non-fatal cardiovascular events and total mortality in type 2 diabetes mellitus. Firstly, it is concluded that skin autofluorescence was able to identify a patient group with a more increased event rate within the group of patients with a high risk for a fatal cardiovascular event of more than 10% within the next 10 years, calculated with the UKPDS risk-engine. The patientgroup with a UKPDS risk score >10% and a skin AF value above the median, had a significantly higher 10-year event rate of 55.8%, compared to group with a UKPDS risk score >10% and a skin AF below the median (38.9%). Since the large majority (79%) of this well-controlled primary-care cohort with relatively recent onset of type 2 diabetes study population has a UKPDS risk score of >10%, it is clinically helpful to have an extra clinical tool to further differentiate the risk in the high risk group. Secondly, when adding skin AF categories (below and above the median) for risk reclassification of patients with UKPDS risk scores of <10%, substantial numbers of patients (27%) would be reclassified to a higher risk group. The incidence of cardiovascular events in patients with a low risk calculated with the UKPDS risk score was 59% higher if they had an AF above the median compared an AF below the median. Reclassification of patients from low to high risk by AF seems, therefore, justified and should result in reconsideration of treatment strategies by extending or intensifying treatment in such individuals. Skin autofluorescence should not result in reclassification of patients from high risk (UKPDS-risk score >10%) to low risk when skin AF is below the median, because the incidence rate of cardiovascular events is high in this group and 'undertreatment' is unwanted.

A history of macrovascular disease and an UKPDS risk score above 10% were significant predictors in the multivariate Cox-regression for all endpoints, and had the highest hazard ratios. The AUC of the Cox-regression analysis in the first model with the UKPDS risk score increased relevantly (>0.025) by addition of skin autofluorescence, which was not statistically significant for the primary endpoint due to the sample size. For the endpoint 'all-cause mortality' the increment of AUC of the

model with AF was higher and significant. In model 2 where a history of macrovascular disease was also included, the addition of AF was not relevant for the primary endpoint and just relevant for the secondary endpoint. The small contribution of AF in this model might be due to the relation of AF with macrovascular disease. In our previous study with cross-sectional data we already showed the relation between AF and clinical apparent vascular damage like a history of macrovascular disease [7]. The present follow-up study confirms skin AF to be a marker of preclinical apparent vascular damage because the incidence of CV-events has increased in the patient group with a baseline skin AF value above the median.

The contributions of skin AF to all models were higher for the secondary endpoints, where 'all cause mortality' was included. This suggests that skin AF or AGEs might also be involved in non-cardiovascular causes of death such as cancer.

A long term follow-up study in a Finnish type 2 DM population, showed that serum levels of AGEs were predictive for all cause and cardiovascular mortality [16]. Besides the papers of this Finnish diabetes cohort, original papers about the predictive value of AGEs for cardiovascular morbidity or mortality are scarce. However, the mechanisms how AGEs can result in vascular injury have been described in the past in extension [1, 17, 20]. A recent study showed a pathophysiological example of the role of AGEs in cardiovascular disease by finding increased myocardial AGE depositions in patients with diabetes and heart failure with reduced left ventricular ejection fraction [18].

Despite our inclusion of patients with a history of CHD (which is different from the UKPDS study), the expected 3.2-year UKPDS-score event-rates in our group were in line with the observed rates, confirming that the UKPDS scores were well applicable and calibrated for our group. The incidence of new CV-events in our group was twice as high in the 20% patients with a history of CHD, compared to patients without a history of CHD. Including patients with a history of CHD seems to be justified by daily practice where a history of CHD is common in even newly diagnosed type 2 diabetes. The UKPDS risk-engine was developed in a young (25-65 years, mean age at diagnosis 52 years) newly-diagnosed type 2 diabetes group without previous cardiovascular disease, detected between 1977 and 1991, selected for participating in a clinical trial,

and using the mean of HbA1c, BP and lipid levels in the first 2 years after diagnosis [9]. Despite the increased incidence at younger ages, the presentation of type 2 diabetes involves mainly subjects over 60 years of age in the Netherlands, often with previous CHD and already treated with statins or antihypertensives. When in such treated patients blood pressure or cholesterol values are put in the UKPDS risk-engine, the calculated risk score might be an underestimated risk. In those patients the clinician is expected to identify those at the highest CV-risk to adapt treatment targets. Our older patient group with HbA1c-levels comparable to that of the UKPDS cohort but with prevalent (39%) macrovascular disease, reflects the differences between our 'daily practice' type 2 diabetes cohort and the UKPDS cohort.

In the present study, no additional contribution of HbA1c was found as predictor for any of the endpoints. The contribution of HbA1c to increased CV-risk in type 2 diabetes was illustrated in the UKPDS by an almost twofold increase in 10-year CHD risk, and by a higher odds ratio for fatal to non-fatal events, both myocardial infarction and stroke [9,19]. This seeming discrepancy with our models including skin AF may be influenced by the relatively low mean HbA1c in our population, but another explanation is the following concept. First, skin AF and skin biopsy AGEs may reflect the impact of the history of hyperglycaemic episodes in diabetes and preceding impaired glucose tolerance, rather than the short-term measure of hyperglycaemia by HbA1c (weeks). Second, AGEs are also formed during oxidative stress via the pathway of reactive carbonyl compound formation [20]. An earlier study found skin AF to be related to CRP and inversely related to antioxidant levels, addressing that skin AF also represent inflammatory along with hyperglycaemic episodes [21]. The DCCT-EDIC group that skin collagen AGE and collagen-linked fluorescence from skin biopsies were better predictors than HbA1c of diabetic complications in type 1 diabetes mellitus [2,3]. They introduced 'metabolic memory', the long-term (years) memory, of stable AGEs bound to long-lived proteins like skin collagen, for episodes of hyperglycaemia and oxidative stress as an explanation for the superiority of skin AGE as a risk predictor. The recent long-term follow-up of glucose control in the UKPDS cohort, showed in the intensive therapy group a continued reduction in risk for microvascular

complications, myocardial infarction and all-cause mortality during 10 years of post-trial follow-up. This is despite the early loss of glycemic and HbA1c differences, and further support to the above concept [22].

Some limitations of our study should be kept in mind: the follow-up period is relatively short. Our calculations were all based on single measurements. Effects of regression dilution bias cannot be excluded. As for the comparison with the UKPDS risk score, in the design of the UKPDS the years 0-4 were excluded from the analysis, since in the first years of the clinical trial mortality rates were lower. We used a cohort which had been recruited and followed for at least 3 years before the baseline AF measurements. Furthermore, our population size was small compared to larger studies who are specifically designed to develop risk-prediction models. Like described in the method section, this study was not powered to reach significance of the clinically relevant increase of AUC of 0.025 by skin AF. However, relevant increase in AUC became visible in this small cohort in this stage of the follow-up. Finally, our results were obtained in a predominantly Caucasian group. AF measurements in dark skinned persons were not considered reliable using the prototype device in our study. Thus, the predictive results of skin AF may not directly be extrapolated to groups with darker skin, but probably need additional validation with newer versions of the AGE Reader.

In conclusion, non-invasive skin AF is a clinical tool which could be used in addition to the UKPDS risk score to identify diabetic subjects with preclinical vascular damage who have a particularly high risk for developing cardiovascular events. A high skin AF value can also result in reclassification to a high risk group in patients who are classified as ‘low-risk’ according to the UKPDS risk-engine. Although many type 2 diabetes patients are nowadays controlled tightly according to guidelines for their classical risk factors, they still develop micro- and macrovascular complications. Therefore, there is a need for additional markers to identify patients at high risk for complications. Furthermore, risk calculators might underestimate cardiovascular risk in patients who are already treated with blood pressure or lipid lowering drugs. Improvement of risk prediction strategies is important to further reduce the incidence or progression of complications in patients with diabetes.

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CHAPTER 6

Normal life expectancy in a large subset of type 2 diabetes patients treated in primary care (ZODIAC-10)

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ABSTRACT

Introduction Earlier studies showed increased mortality in individuals with type 2 diabetes mellitus. As a result of major changes in treatment regimes over the past years, with more stringent goals for metabolic control and cardiovascular risk management, improvement of life expectancy should be expected. In our study, we aimed to assess present-day life expectancy of type 2 diabetes patients in an ongoing cohort study.

Methods We included 973 primary care type 2 diabetes patients in a prospective cohort study who were all participating in a shared care project in the Netherlands. Vital status was assessed from May 2001 until May 2007. Main outcome measurement was life expectancy assessed by transforming survival time to standardised survival time allowing adjustment for the baseline mortality rate of the general population.

Results At baseline, mean age was 66 years, mean HbA1c 7.0%. During a median follow-up of 5.4 years, 165 patients died (78 from cardiovascular disease) and 17 patients were lost to follow-up. There was no difference in life expectancy of type 2 diabetes patients compared to the life expectancy of the general population. In multivariate Cox regression analyses, concentrating on the endpoints 'all-cause' and cardiovascular mortality, a history of cardiovascular disease: HR 1.71 (95% CI 1.23-2.37), and HR 2.59 (95% CI 1.56-4.28), and albuminuria: HR 1.72 (95 %CI 1.26-2.35) and HR 1.83 (95%CI 1.17-2.89), respectively, were significant predictors, whereas smoking, HbA1c, systolic blood pressure and diabetes duration were not.

Conclusion This study shows a normal life expectancy in type 2 diabetes patients in primary care. A history of cardiovascular disease and albuminuria, however, increase the risk of a reduction of life expectancy. These results show that, in a shared care environment, a normal life expectancy is achievable in type 2 diabetes patients.

INTRODUCTION

The incidence and prevalence of diabetes mellitus has risen worldwide during the past few decades. Recently published data from the Framingham Heart Study showed an absolute increase in the incidence of diabetes of ~ 2.5% during the 1990s compared to

the 1970s [1]. The proportion of cardiovascular disease attributable to diabetes mellitus has increased as well [2]. Other studies over the last decades of the previous century also showed increased mortality rates in diabetes mellitus compared to the general population, mostly due to cardiovascular events [3-7]. However, since the introduction of new pharmaceutical therapies and more stringent regimens for the treatment of hyperglycaemia, hypertension, dyslipidaemia, and other cardiovascular risk factors, trends in reduction of (cardiovascular) mortality rates amongst diabetic patients have been seen [8-12]. This improvement of survival could be expected to be comparable to the decrease in cardiovascular mortality rates in the general population due to aggressive management of cardiovascular risk factors as well.

Most published data were extracted from representative national cohorts in North-America or the United Kingdom. Some reports showed a decline in mortality rates amongst diabetic men only, whereas women showed an increase or no change in mortality rates at all [4,8,11,12]. A recently published Scandinavian study showed a substantial decrease in mortality rates from coronary heart disease in all age groups irrespective of sex and diabetes status over two consecutive time periods. However, the more than twofold higher mortality from coronary heart disease in diabetes patients compared to the non-diabetic population remained over time. This finding also suggest a longer survival in diabetes patients resulting from intensified treatment of cardiovascular risk factors [13].

Our aim was to investigate present-day life expectancy in a type 2 diabetes population treated in a primary care, who also received additional support in a shared care setting in a West-European country.

METHODS

Subjects In 2001, 973 type 2 diabetes patients participated in a cross-sectional study with measurements of skin advanced glycation endproduct (AGE) accumulation as described previously [14]. This study was embedded in a long-term shared care project (ZODIAC: Zwolle Outpatient Diabetes project Integrating Available Care) concerning

a primary care treated population-based sample of type 2 diabetes patients in an eastern district of The Netherlands. *Figure 2* of Chapter 1 (page 15) shows an overview of the enrolment of the current study cohort started from the beginning of the ZODIAC. During this project, 32 general practitioners (GPs) were supported by hospital diabetes specialist nurses and consultant-physicians [15]. In short, all type 2 diabetes patients were exclusively treated by their GPs and visited the diabetes specialist nurses for evaluation of metabolic control and diabetes related complications annually. After these evaluations, treatment advice for individual patients as well as for benchmarking was given to the GPs by internists in the Isala Clinics in Zwolle, the Netherlands. Advice and referrals were based on guidelines of the Dutch College of General Practitioners [16]. Patients with a cognitive disability or terminal disease were not included in the ZODIAC study because of their inability to undergo educational programs. Furthermore, patients who were physically unable to visit the diabetes specialist nurse at the outpatient clinic were not enclosed in the present cohort. This study was approved by the local ethical committee of the Isala Clinics, Zwolle, The Netherlands and all patients gave written informed consent.

Procedures Methods of clinical data collection and laboratory assessments have been described in detail elsewhere [14]. Before participation in our study, diagnosis of diabetes mellitus was already made in individuals with fasting plasma glucose levels ≥ 7.0 mmol/liter. The following definitions representing the diabetic complications at baseline are: retinopathy, which was defined as the presence of at least background retinopathy or a history of laser coagulation for diabetic retinopathy. Albuminuria was defined as an albumin-to-creatinine ratio >3.5 mg/mmol for women or >2.5 mg/mmol for men. Diminished sensibility at least at one foot was considered as neuropathy, tested with a 5.07 Semmes-Weinstein monofilament, applied on three areas of each foot [16]. The presence of microvascular disease was defined as meeting the criteria of retinopathy, albuminuria, and/or neuropathy. The presence of cardiovascular disease at baseline was defined when meeting at least one aspect of cardiovascular disease: ischemic heart disease (IHD), International Classification of Diseases ninth revision

(ICD-9), codes 410-414 and/or a history of coronary artery bypass surgery or percutaneous coronary intervention, cerebrovascular accidents including transient ischemic attacks (CVAs) and/or peripheral vascular disease (PVD). PVD is defined as surgical intervention, claudication and/or absent pulsations of ankle or foot arteries (absence of pulsations of the dorsalis pedes arteries bilaterally was not scored as PVD when tibial artery pulsations were present).

Mortality was registered from the date of inclusion until May 2007. Death was certified according to the following procedure. In addition to the list of deceased patients reported in the files of the scheduled annual follow-up visits, survival status of the patients was obtained from the local hospital information system and verified with the GPs. Date of death was collected likewise. None of the GPs had involvement or interest in study outcome. Causes of death were coded according to ICD-9 and categorised as: neoplasms (140 – 239), diseases of the cardiovascular system (390 – 459), diseases of the respiratory system (460 – 519), diseases of the digestive system (520 – 579), diseases of the genitourinary system (580 – 629), injury and poisoning (800 – 999). Sudden death, with symptoms present less than one hour, was encoded in the category of coronary heart disease. For the in-hospital deaths, the medical records were retrieved. For the out-of-hospital deceased patients, the assigned causes of death by the GPs were obtained from the medical records of the GPs. The coded causes of death were combined to all-cause mortality (all codes) and cardiovascular mortality (390 – 459 or sudden death).

Statistical analyses Analysis of life expectancy was performed primarily by the use of ‘standardised survival time’ (SST), which is a novel approach to survival analysis [17]. SST is another expression of follow-up time than survival time in years. This method provides survival time which is adjusted for the median residual life span of individuals in the general population with the same age and sex. Due to this standardisation of survival time there is no influence of the interactions between age and the presence and effects of other risk factors. Furthermore, it allows assessment of the effectiveness of treatment on regaining a normal residual life span. SST was calculated as the ratio

between the observed survival time of an individual and the median residual life span of individuals with the same age and sex in the general population at the starting date of the study. The median residual life span was derived from gender specific reports provided by the Dutch Central Office of Statistics, which is the national institution of statistics and demographics [18]. Direct comparisons between study samples and the general population were done by comparing the 95% confidence interval (CI) of each median SST with an expected value of 1. The 95% CI of mortality at a SST of 0.25 and 0.5 were calculated and compared with the expected mortality as calculated for the age and gender matched general population, assuming Poisson distribution of the events. Kaplan-Meier curves were constructed for survival and for standardised survival. A Cox proportional hazard model to estimate hazard ratios (HR) and 95% CI was used in the standard way using survival time in years, and additionally by using SST. Methodologically, it is allowed to use SST instead of survival time in years in a Cox-regression model, as it is consistent with the preconditions of a Cox regression analysis: an increase in mortality and an increase in follow-up time have to be present. P values <0.05 were considered to be statistically significant. Clinical and laboratory variables with an expected risk for mortality were first analysed in a univariate analysis, and secondly, in a multivariate model. Detailed analyses were done for two end-points: all-cause mortality and cardiovascular mortality.

RESULTS

Characteristics of the 973 type 2 diabetes patients at baseline (2001) are shown in *Table 1*. The population had a relatively short median diabetes duration of 4.2 (interquartile range 1.6 – 8.3) years and on average a good glycemic control (mean HbA1c 7.0%). *Table 1* also shows the baseline characteristics of patients when subdivided in survivors (791 patients) and non-survivors (165 patients). At the end of a median follow-up duration of 5.4 years (interquartile range 5.1 – 5.6), 165 patients had died (17%) and 17 patients were lost to follow-up.

Table 1. Baseline characteristics of type 2 diabetes patients: total and subdivided in survivors and non-survivors, expressed as mean ± SD or n (%)

Characteristic	*Total (N = 973)*	*Survivors (N=791)*	*Non-survivors (N=165)*	*p-value*
Age in years	66.4 (11.3)	64.8 (11.1)	73.6 (9.4)	<0.001
Male gender (%)	47	46.3	50.9	0.278
Smoking (%)	19.3	19.3	20.6	0.71
Body mass index in kg/m^2	29.38 (4.87)	29.5 (4.8)	28.7 (5.1)	0.077
Systolic blood pressure in mmHg	146.01 (20.15)	145 (20)	149 (20)	0.015
Diastolic blood pressure in mmHg	81.18 (10.34)	81 (10)	80 (11)	0.046
Diabetes duration in years	[a]4.16 (1.62-8.31)	[a]3.92 (1.50-8.04)	[a]5.03 (2.40-10.8)	0.002
HbA1c (%)	6.96 (1.3)	6.95 (1.32)	6.998 (1.23)	0.69
Creatinine in µmol/l	96.0 (19.88)	94.56 (17.6)	103.16 (27.69)	<0.001
Creatinine clearance in ml/min	76.13 (26.91)	78.8 (26.6)	63.22 (24.75)	<0.001
Urinary albumin-to-creatinine ratio	[a]1.49 (0.80-4.17)	[a]1.35 (0.75-3.44)	[a]3.09 (1.23-11.01)	0.001
Total cholesterol in mmol/l	5.16 (1.01)	5.17 (1.02)	5.08 (1.00)	0.302
Cholesterol-to-HDL ratio	4.34 (1.23)	4.37 (1.21)	4.22 (1.36)	0.171
HDL cholesterol in mmol/l	1.25 (0.33)	1.24 (0.32)	1.29 (0.35)	0.141
LDL cholesterol in mmol/l	2.87 (0.93)	2.85 (0.92)	2.92 (0.98)	0.388
Triglycerides in mmol/l	2.32 (1.36)	2.39 (1.40)	2.03 (1.14)	0.002
Microvascular disease (%)	54.1	50.2	70.9	<0.001
Retinopathy (%)	19.6	18.5	24.8	0.050
Microalbuminuria (%)	25.5	21.4	45.5	<0.001
Neuropathy (%)	29.1	26	40.6	<0.001
Cardiovascular disease (%)	39.5	34.6	63.6	<0.001
Ischemic heart disease (%)	21.5	19.6	30.3	0.002
Cerebrovascular disease (%)	7.8	6.4	14.5	<0.001
Peripheral vascular disease (%)	23.0	18.1	47.3	<0.001
RAS-inhibitors[b] (%)	37.2	36.5	39.4	0.489
in patients with IHD (%)	42.1	38.7	50	0.159
in patients with PVD (%)	46.9	44.1	50	0.397
Lipid-lowering drugs[c] (%)	29.8	30.7	26.1	0.234
in patients with IHD (%)	57.4	61.9	44	0.026
in patients with PVD (%)	32.6	33.6	29.5	0.535
Antiplatelet drugs (%)	24.9	22.1	38.2	<0.001
in patients with IHD (%)	67.5	68.4	64	0.565
in patients with PVD (%)	40.2	35.7	46.2	0.127
Diabetes treatment – Diet only (%)	20.2	21.4	16.4	
Oral medication	64.1	64.3	63	
Insulin (%)	9.8	8.3	15.8	
Both (%)	5.9	5.9	4.8	

Seven patients were lost to follow-up and did not define the baseline characteristics of the survivors/non-survivors. [a]Median and interquartile range, [b]Angiotensin-converting enzyme inhibitors and Angiotensin II receptor blockers, [c]Large majority represented by statins (99%). Reference values of the laboratory: HbA1c 4.0-6.0 %, creatinine 70-110 µmol/l, creatinine clearance (Cockcroft-formula) 80-120 ml/min, urinary albumin-to-creatinine ratio 0-2.5 for men and 0-3.5 for women, total cholesterol 3.5-5.0 mmol/l. Abbreviations: IHD, ischemic heart disease; PVD, peripheral vascular disease.

Minimum follow-up duration of all survivors was 5.0 years. Ten persons of the losts to follow-up have had a last visit to the outpatient clinic between baseline and the end of follow-up; this last registered visit date was determined as the end of follow-up of these patients. The remaining 7 persons lost to follow-up had a mean age of 68 years, were non-smokers, and 2 patients had cardiovascular disease at baseline. Their median diabetes duration was 9.7 years with a mean HbA1c of 7.5 %.

The proportion of prescribed lipid-lowering drugs, renin-angiotensin system (RAS) inhibitors and antiplatelet therapy at baseline, is shown at the end of *Table 1*. The percentages prescribed lipid-lowering drugs and antiplatelet drugs were significantly higher in patients with a history of IHD compared to the patients with PVD ($p<0.001$). At baseline, antiplatelet drugs were significantly more prescribed in the non-survivors compared to the survivors. By contrast, in patients with ischemic heart disease lipid lowering drugs were significantly less prescribed in the non-survivors compared to the survivors.

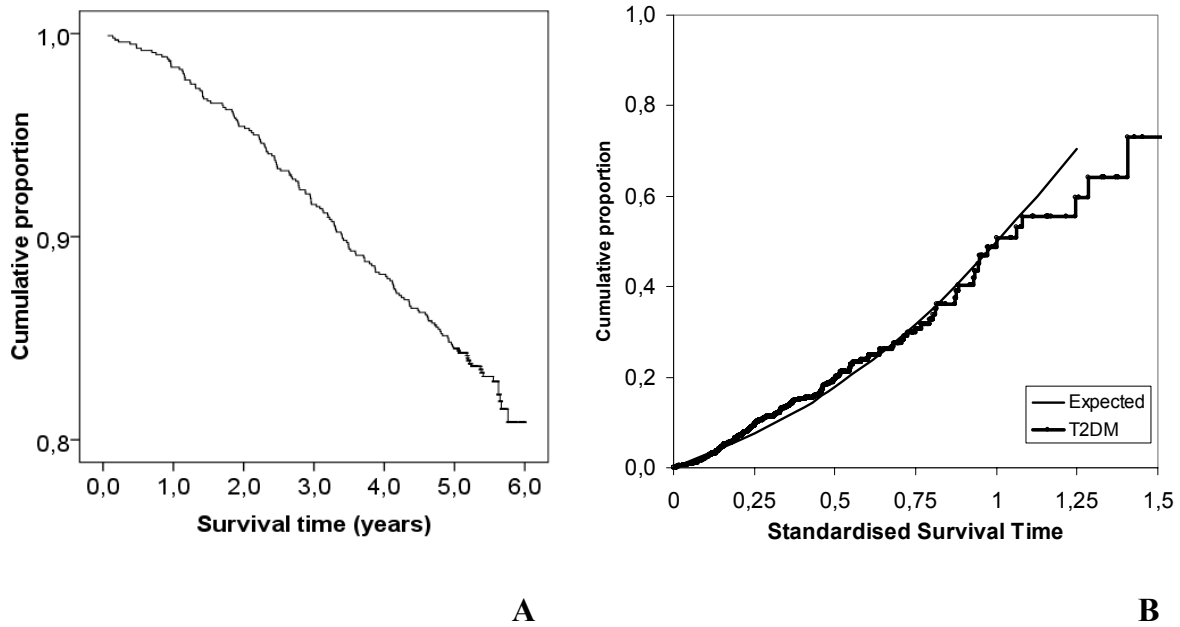

Figure 2. Kaplan-Meier survival curve for survival in years in the entire type 2 diabetes group (**A**) and Kaplan-Meier survival curve for survival expressed as Standardised Survival Time (SST) in the entire type 2 diabetes group (**B**). The median SST in type 2 diabetes mellitus (T2DM) 1.00 is not different from the general Dutch population 1.00 (Expected); the observed mortality (all-cause) at SST 0.25 and 0.50 of 0.09, respectively 0.20 does not significantly differ from the general Dutch population (0.08 respectively 0.18 , $p>0.1$).

The proportion of cardiovascular deaths, 47%, was increased when compared to the general Dutch population. In 2007, 31% of all deaths in the general Dutch population were due to cardiovascular disease, with a highest relative incidence of 38% cardiovascular deaths in the population above 85 years [18].

Figure 2A shows the Kaplan-Meier curve of the cumulative proportion of survivors in our type 2 diabetes population against survival time in years. A Kaplan-Meier plot of the cumulative proportion of deaths in our study population against standardised survival time is shown in *Figure 2B*; the expected mortality for the age- and gender-matched general population is also shown. The median standardised survival time in our study population was 1.00 [95 % confidence interval (CI) 0.88 – 1.12] and did not differ from the general population. The cumulative proportion of deaths at half standardised survival time (SST = 0.50) was 0.20 (95 % CI 0.16 – 0.23), which did not differ from the expected value of 0.18 in the general population.

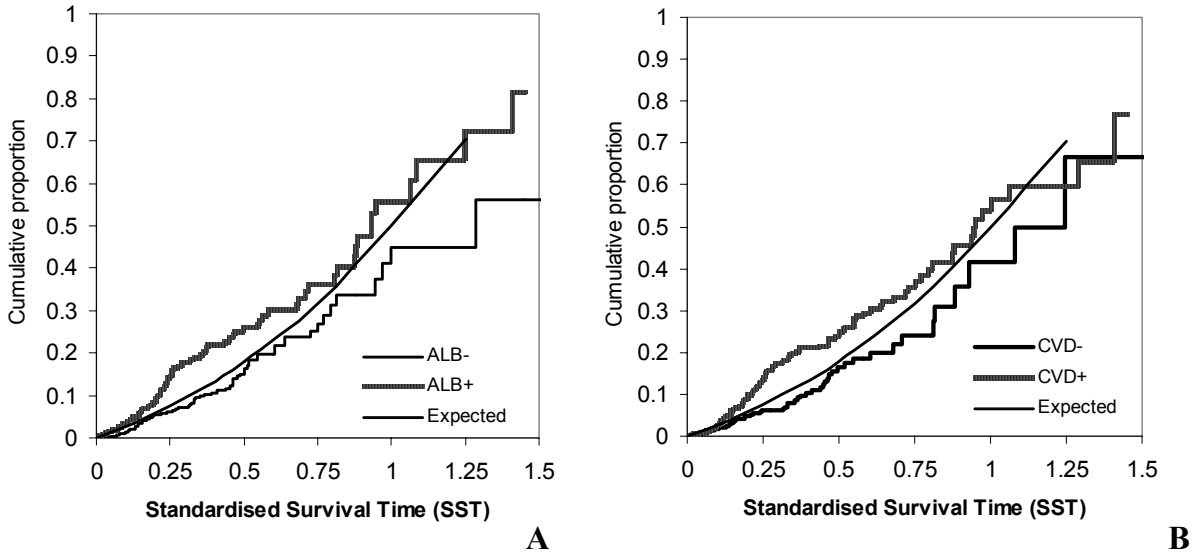

Figure 3. Cumulative proportions of deaths against Standardised Survival Time (SST) in patients with (**A**) albuminuria (Alb) yes/no (+/-) and (**B**) previous cardiovascular disease (CVD) yes/no (+/-), compared to the expected deaths of the general population. Differences in mortality between the diabetes-subgroups and the general population are tested at SST= 0.25 and SST=0.5. For patients with albuminuria, mortality rate at SST 0.25 is 0.15 (95 % CI 0.10-0.19) and the expected value is 0.076 (p=0.002). At SST 0.50 mortality rate is 0.26 (95 % CI 0.19-0.33), which was also higher than the expected value of 0.18 (p=0.014). For patients with a history of cardiovascular disease (CVD) mortality rate at SST 0.25 is 0.13 (95% CI 0.096-0.17) and an expected value is 0.076, p<0.001. At SST 0.50 mortality rate is 0.25 (95% CI 0.19-0.30) and the expected value is 0.18, p<0.0001.

Figure 3A shows a mortality rate in the type 2 diabetes patients with albuminuria at SST 0.25 of 0.15 (95 % CI 0.10 – 0.19), which was higher than the expected value of 0.076 (p=0.002). At SST 0.50 mortality rate was 0.26 (95 % CI 0.19 – 0.33), which was also higher than the expected value of 0.18 (p=0.014). This also holds for the type 2 diabetes patients with a history of cardiovascular disease, who had a higher mortality at SST 0.25 [0.13 (95% CI 0.096 – 0.17), p<0.001] and SST 0.50 [0.25 (95% CI 0.19 – 0.30), p<0.0001], *Figure 3B*.

Table 2. Predictors of overall mortality in type 2 diabetes mellitus by univariate and multivariate Cox regression analysis using "survival in years" (= standard method) and using "standardised survival time"

Predictors of all-cause mortality	*Survival in years*			*Standardised survival time* [a]		
	Univariate					
	HR	*95% CI*	*p-value*	*HR*	*95% CI*	*p-value*
Gender (man = reference)	0.84	0.62 – 1.14	0.25	0.91	0.67 – 1.24	0.56
Age	1.08	1.06 – 1.10	<0.001	1.00	0.98 – 1.02	0.90
Smoking	1.10	0.75 – 1.60	0.63	1.49	1.02 – 2.18	0.039
Systolic blood pressure	1.01	1.00 – 1.02	0.016	1.00	0.99 – 1.01	0.66
Diabetes duration	1.03	1.02 – 1.05	0.001	1.02	1.00 – 1.04	0.073
HbA1c	1.02	0.91 – 1.15	0.71	1.08	0.95 – 1.22	0.23
Albuminuria (yes/no)	2.33	1.71 – 3.17	<0.001	1.81	1.32 – 2.46	<0.001
History of cardiovascular disease (yes/no)	2.95	2.15 – 4.06	<0.001	1.87	1.35 – 2.58	<0.001
Use of lipid-lowering drugs (yes/no)	0.83	0.59 – 1.18	0.30	1.34	0.80 – 1.62	0.48
Use of antiplatelet drugs (yes/no)	2.00	1.46 – 2.74	<0.001	1.47	1.07 – 2.01	0.018
			Multivariate			
Gender (man = reference)			NS			x
Age	1.07		<0.001	x	x	x
Smoking (yes/no)		1.05 – 1.09	NS	x	x	NS
Systolic bloodpressure			NS			NS
Diabetes duration			NS			NS
HbA1c			NS			NS
Albuminuria (yes/no)	1.79		<0.001			0.001
History of cardiovascular disease (yes/no)	1.79	1.30 – 2.46	0.001	1.72	1.26 – 2.35	0.001
Use of lipid-lowering drugs (yes/no)		1.29 – 2.50	NS	1.71	1.23 – 2.37	NS
Use of antiplatelet drugs (yes/no)			NS			NS

HR=hazard ratio; CI=confidence interval; NS=not significant. [a] The standardised survival time was calculated as the ratio between the observed survival time of an individual and the median residual life span of individuals with the same age in the general population.

Table 2 shows the hazard ratios (HRs) and 95% CI of univariate and multivariate Cox-regression analyses for all-cause mortality. The HRs in the univariate analyses are higher for all cardiovascular disease items compared to the method of using SST in the model. In the multivariate analysis, predictive factors for all-cause mortality were comparable for both methods when age and gender were included in the model of the standard method: a history of cardiovascular disease (HR 1.79 and 1.71) and, albuminuria (HR 1.79 and 1.72). Univariate analysis of the endpoint: cardiovascular mortality (not shown in *Table 2*) resulted in the same significant predictive factors, but with higher HRs. Multivariate analysis of cardiovascular mortality resulted in the same significant predictive factors with higher HRs (SST) as well: albuminuria 1.83 (95% CI 1.17 – 2.89); history of cardiovascular disease 2.59 (95% CI 1.56 – 4.28). Smoking, systolic bloodpressure, diabetes duration and HbA1c were not significant in both multivariate models.

DISCUSSION

This study shows a normal median overall life expectancy in a cohort of type 2 diabetes patients treated in a primary care setting, during a follow-up period from 2001 until 2007. This finding implies that the current available treatment strategies are effective in supporting the opportunities for a life expectancy equal to the general population in this subset of type 2 diabetes patients. Secondly, in this type 2 diabetes study population, patients with a history of cardiovascular disease and/or the presence of albuminuria had an increased risk to die before their median life expectancy was reached. The differences in effects of all items of cardiovascular disease on ‘survival in years’ and SST, could be explained by the age correction enclosed in the SST – method. As the prevalence of cardiovascular diseases is increasing during aging, SST seems to be preferable to the ‘classical survival time’ in identifying premature mortality.

Finally, we still found an increased proportion of deaths due to cardiovascular disease compared to the general population (47% versus 31%). This is in agreement with

established observations of increased cardiovascular disease in diabetes, and also with the presence of cardiovascular risk factors we found to be most intimately related to reduce life expectancy [13,19].

The United Kingdom Prospective Diabetes Study reported a 5 years reduction of life expectancy for males aged 45 to 50 years at diagnosis of diabetes when compared to the general United Kingdom population [6]. Estimations of reduction of life expectancy for patients with diabetes diagnosed at an older age are not presented explicitly in this paper, but might be smaller than 5 years, as other studies showed that reduction of life expectancy decreases with diagnosis at older age [5,12,20].

A large study of the noninstitutionalised United States population, which was conducted between 1971 and 1993, showed a median reduced life expectancy of 8 years for the diabetic population aged 55-64 years, and a 4 years reduction for the diabetic population aged 65-74 years [4]. However, these studies were all executed in a period during which treatment with statins, angiotensin-converting enzyme inhibitors and antiplatelet medication was much less common practice. A more recent study, showing slightly increased mortality in women but no excess mortality in men, included exclusively patients diagnosed with type 2 diabetes mellitus over the age of 65 [12]. Our study is of additional value, as we included primary care type 2 diabetes patients of all ages, representing a large amount of the type 2 diabetes patients in the Netherlands who are mostly treated in primary care according national guidelines. Sixty-four percent of our study population was diagnosed with type 2 diabetes mellitus before the age of 65 years.

A previous study in the first ZODIAC-cohort (1998) reported an annual mortality rate of 4.8% between 1998 and 2000, which was higher than the mortality rate in the current study (~3%), whilst continuing the follow-up in this shared care environment [21]. This difference could be explained by the fact that the earlier analysis was performed in a more extended type 2 diabetes cohort, which also included patients who were referred to secondary care. It is also possible that the cohort as presented in this earlier study had to benefit yet from participation in a long-term shared-care environment with supportive care and monitoring of implementation of the guidelines.

More than half of our population received either a statin, RAS-inhibitor or aspirin at baseline. At follow-up, this proportion was increased to at least 80%. Widespread treatment of the traditional cardiovascular risk factors resulted in vastly improved blood pressure readings and lipid levels. This could also be the explanation for disappearance of systolic blood pressure from the model to predict mortality. Recent studies addressed the importance of statins and blood pressure lowering drugs in patients with type 2 diabetes mellitus, showing a reduction in cardiovascular events with these lipid-lowering drugs compared to placebo [22-24].

Remarkably, we observed a lower proportion of prescribed lipid-lowering drugs and antiplatelet drugs in the PVD patients of our total cohort, compared to those with IHD. This prescription behaviour was reported before [25]. In general, IHD has a more clear and urgent manifestation and is therefore diagnosed in an earlier phase. Patients with PVD are often asymptomatic and clinicians are less focused on screening and the treatment of PVD and its associated risk factors [26,27]. Furthermore, we observed a lower proportion of prescribed lipid-lowering drugs in patients with IHD who did not survive. This suggests the beneficial role of these drugs on mortality. The higher proportion of antiplatelet drug use at baseline in the non-surviving group and its risk on mortality in univariate analyses remained unexplained.

HbA1c had also no effect on life expectancy in uni- and multivariate analysis. One might assume that this may be explained by the low number of patients with poor glycemic control (only 7% had a HbA1c >9%). Alternatively, other mechanisms could be involved in the development of diabetes related complications. Recently, we reported increased levels of advanced glycation endproducts (AGEs) in the same study group, which were related to chronic complications [14].

Patients who were referred to the secondary care in the past, mainly for reasons of poor metabolic control or comorbidity, were not included in this study and could have a reduced life expectancy. Also, there will be a selection bias by excluding patients with a very short life expectancy (terminally ill patients, cognitive disabled people and patients who are unable to undergo educational programs), as described in the methods section. Still, 40% of the included study population suffered from cardiovascular

disease at baseline. Despite the apparent selection bias, we did have a large subset of patients with a life expectancy comparable to that of the general population of the same age and sex. Furthermore, in The Netherlands, the large majority (70 – 80%) of type 2 diabetes patients is treated in primary care or a shared-care setting. Therefore, this study population could be representative for the majority of type 2 diabetes patients in The Netherlands, and probably also for a larger part of type 2 diabetes patients in other countries with structured diabetes care.

Our choice to compare life expectancy of this type 2 diabetes cohort to the general population could be criticised, because the general population also includes people with diabetes, cardiovascular disease, cancer, and other life shortening diseases. We nevertheless preferred to choose the general population instead of a non-diabetic control group, because one of the aims of caregivers within the medical community is to regain a life expectancy for their patients equal to the general population when life expectancy is reduced due to a specific disease.

To visualise whether a life expectancy equal to the general population had been achieved, we used SST. Traditional survival analysis focuses more on 'mortality' within a certain follow-up time, but with this method it is not clear whether it is 'normal mortality' or 'excess mortality'. Using SST, the mortality rate is adjusted for the median survival of subjects in the general population of the same age and sex. Excess mortality or a reduced life expectancy will be identified more easily in that way.

The results of this study are relevant for clinical practice, because they offer a hopeful perspective of a definitely improved life expectancy in type 2 diabetes patients. We suggest that those results are also (partly) due to the fact that these patients were and are participating in a care system promoting adherence to evidence-based guidelines and strong lines of cooperation between health care providers focusing on this patient group. Our study once more confirms the implications of the presence of cardiovascular disease and the diagnosis of albuminuria on mortality risk in type 2 diabetes mellitus.

In summary, this study shows a normal life expectancy in a large subset of type 2 diabetes patients treated in a primary care setting. The presence of previous cardiovascular disease and albuminuria, however, can markedly reduce life expectancy.

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CHAPTER 7

Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications

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ABSTRACT

Introduction Skin autofluorescence (AF) is a noninvasive measure of the level of tissue accumulation of advanced glycation endproducts, representing cumulative glycemic and oxidative stress. Recent studies have already shown a relationship between skin AF and diabetic complications, and its predictive value for total and cardiovascular mortality in type 2 diabetes mellitus. Our aim was to investigate the predictive value of skin AF for the development of microvascular complications in type 2 diabetes mellitus.

Methods At baseline, skin AF of 973 well-controlled type 2 diabetes patients was noninvasively measured with an AF reader. The aggregate clinical outcome was defined as the development of any diabetes associated microvascular complication of 881 surviving patients which was assessed at baseline and at the end of follow-up. Single endpoints were the development of diabetes associated retinopathy, neuropathy and (micro)albuminuria.

Results After a mean follow-up period of 3.1 years, baseline skin AF was significantly higher in patients who developed any microvascular complication, neuropathy and (micro)albuminuria, but not in those who developed retinopathy. Multivariate analyses showed skin AF as a predictor for the development of any microvascular complication along with HbA1c; for the development of neuropathy along with smoking, and for the development of (micro)albuminuria together with gender, HbA1c and diabetes duration. Skin AF did not have predictive value for the development of retinopathy, albeit diabetes duration did.

Conclusion Our study is the first observation of skin AF measurement as an independent predictor for the development of microvascular complications in type 2 diabetes mellitus.

INTRODUCTION

Hyperglycaemia, individual susceptibility and life-style are three key factors, which play an important role in the development of microvascular disease in diabetes

mellitus. One of the consequences of hyperglycaemia and attendantly increased generation of free radicals is the increased formation of advanced glycation endproducts (AGEs), besides the increased polyol and hexosamine fluxes, and activation of protein kinase C, which all contribute to tissue damage in diabetes [1,2]. Those AGEs can be described as the final products of slowly occurring non-enzymatic glycation of proteins that form cross-links with long-lived proteins such as collagen (the so called Maillard reaction). They may also accumulate as a result of oxidative stress related glycoxidation and lipoxidation pathways.

In the DCCT, intensive treatment as compared with conventional treatment showed that long term intensive treatment of hyperglycaemia in type 1 diabetic patients improved glycemic control, and delayed the progression of microvascular complications [3]. The UKPDS and other prospective studies have also shown an association between hyperglycaemia and increased risk of microvascular complications in type 2 diabetes [4-6]. The DCCT skin collagen ancillary study group showed the association of long-term intensive treatment of hyperglycaemia, as compared with conventional treatment, with lower levels of AGEs in skin collagen and they showed that these AGE levels in skin biopsies predicted the risk of development or progression of microvascular disease in type 1 diabetes mellitus, even after adjustment for HbA1c [7,8].

A newly described noninvasive method to assess tissue AGEs concerns skin autofluorescence (AF). This method is based on the specific fluorescence characteristics of AGEs and has been validated against specific AGE levels in skin biopsies in patients with diabetes or on hemodialysis, and in healthy controls [9,10].

Recently, the relationship between skin AF, reflecting AGE accumulation, and outcome has been studied in type 2 diabetes. Besides its relation with chronic complications of diabetes (in cross-sectional analyses), skin AF has also shown its independent predictive value for cardiovascular mortality and morbidity in patients with type 2 diabetes, and in patients with end-stage renal disease undergoing hemodialysis [10-12].

In this study, we analyzed whether skin AF, as a marker of AGE accumulation, can predict the development of microvascular complications in a type 2 diabetes population.

METHODS

Patients. Between May 2001 and May 2002, 973 primary care type 2 diabetes patients were included in the study cohort and had a skin AF measurement. The included patients were all participating in a shared-care project of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC)-study and have also been described elsewhere [11]. During follow-up, data of 967 patients were analyzed for this study (6 patients were lost to follow-up). Eighty-six patients died before the end of follow-up and this subgroup will be addressed separately from the surviving 881 patients. Patients with a Fitzpatrick class V-VI skin type were excluded, because of the limitation of the autofluorescence reader (AFR) to measure accurately in dark skin types [13-15]. All participating patients visited the outpatient clinic at least once a year. Follow-up ended at January 2005. All of the included patients had given their informed consent, and approval by the local ethical committee had been obtained.

Skin autofluorescence. The AFR (prototype of the current AGE Reader; DiagnOptics BV, Groningen, the Netherlands), which measures skin AF, illuminates a skin surface of ~4 cm^2, guarded against surrounding light, with an excitation light source with peak intensity at ~370 nm. Emission light and reflected excitation light from the skin are measured with a spectrometer in the 300-600 nm range, using a glass fiber. AF was computed by dividing the average light intensity of the emission spectrum 420-600 nm by the average light intensity of the excitation spectrum 300-420 nm, multiplied by hundred and expressed in arbitrary units (a.u.). Skin AF of all patients was assessed at the volar side of the arm, 10 cm below the elbow fold. Six diabetes specialist nurses did the AF measurements with 2 identical AFR devices. The AFR has been validated and more extensively been described in previous studies [9,11].

Data collection. Clinical data and laboratory results were obtained at the time of the baseline skin AF measurement. Serum creatinine, nonfasting lipids (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides), and urinary albumin and creatinine were measured according to the standard laboratory procedures. HbA1c was measured with a Primus CLC-385 using boronate affinity chromatography and high-performance liquid chromatography (reference value 4.0 – 6.0%). Blood pressure measurement was a single measurement obtained after 5 minutes rest, with the patient in seated position, and using an aneroid device. At each visit to the outpatient clinic, and at the end of follow-up, the absence or presence of retinopathy, neuropathy and (micro)albuminuria were assessed.

Clinical endpoints. The aggregate clinical endpoint was the development of any diabetes associated microvascular complication, which was defined as the presence of at least one of the following diabetic complications: retinopathy, neuropathy and/or (micro)albuminuria, according the ADA Diabetes definitions [16]. The single clinical endpoints were described as the development of retinopathy, neuropathy or (micro)albuminuria. Retinopathy was determined by an ophthalmologist based on retinal photography. Presence of at least background retinopathy was assumed to imply retinopathy. Neuropathy was examined using a 5.07/10-g Semmes-Weinstein monofilament, applied on the dorsum of both feet at three different, non-callused areas (first toe, and first and fifth distal metatarsal bone). Neuropathy was considered in case of diminished sensibility, which was defined in case of at least two incorrect responses after 3 applications at each area (2 real and 1 false application) [17,18]. (Micro)albuminuria at baseline was defined as an albumin-to-creatinine ratio >2.5 mg/mmol for men and >3.5 mg/mmol for women in 2 subsequent urine samples or once in the year before baseline while using an ACE-inhibitor at baseline [19]. Newly developed (micro)albuminuria at follow-up was defined as an albumin-to-creatinine ratio >2.5 mg/mmol for men and >3.5 mg/mmol for women in 2 urine samples (one in the year before and one at the moment during follow-up) or an abnormal level of the albumin-to-creatinine ratio in the year before the end of follow-up whilst using an ACE-inhibitor at follow-up.

Statistical analysis

Oneway ANOVA using posthoc multiple comparisons (with Bonferroni correction) was used to compare mean skin AF between subgroups of microvascular complications in the 881 surviving patients. Subgroups are: A. no microvascular complication at baseline nor at follow-up. B. no microvascular complication at baseline, but a microvascular complication at follow-up. C. a microvascular complication at baseline and at follow-up.

Univariate and multivariate multinominal regression analyses were performed to determine the relationship of skin AF to the presence or development of microvascular disease. Patients without signs of microvascular complications at baseline nor at follow up were the reference categories in these calculations. In the multivariate analyses we controlled for potential confounding risk factors for the development of microvascular complications which were derived from the UKPDS findings, thereby including: gender, diabetes duration, HbA1c, current smoking, systolic blood pressure, HDL cholesterol, LDL cholesterol and triglycerides with the addition of BMI [4].

Odds-ratios (OR) and confidence intervals (95%) for skin AF were calculated in the univariate and in the multivariate analyses; p-values <0.05 were considered to be statistically significant.

RESULTS

The baseline characteristics of the surviving study population including mean skin AF of the total group are shown in *Table 1*. Mean age of our study population was 66 years, 46% male, with a relatively short median diabetes duration of 4.0 years and an interquartile range between 1.5 and 8.1 years. Eighty-five percent of this well-controlled diabetic study population was on a diet and/or oral agents; the other 15% of patients received insulin or combined insulin/oral agent treatment. In the 881 survivors, the prevalence of retinopathy, neuropathy and (micro)albuminuria at baseline was 19%, 24% and 24% respectively, resulting in an overall percentage of patients with a diabetes associated microvascular complication of 50%.

Table 1. Characteristics of the type 2 diabetic patients

Characteristic	*n=881*
Age in years	66 (11)
Gender (M/F)	406/475
Smoking (%)	19
BMI (kg/m²)	29.4 (4.8)
Systolic blood pressure (mmHg)	146 (20)
Diabetes duration (years)	4.0 (1.5-8.1)*
HbA1c (%)	6.6 (6.0-7.6)*
Creatinine (µmol/l)	95 (19)
Creatinine clearance (Cockcroft-formula) (ml/min)	77 (27)
Urinary albumin-to-creatinine ratio	1.41 (0.76-3.79)*
Total cholesterol (mmol/l)	5.2 (1.0)
HDL cholesterol (mmol/l)	1.3 (0.3)
LDL cholesterol (mmol/l)	2.9 (0.9)
Triglycerides (mmol/l)	2.1 (1.4-2.9)*
Microvascular disease (%)	50
Retinopathy (%)	19
Neuropathy (%)	24
(Micro)albuminuria (%)	24
Macrovascular disease (%)	37
Skin autofluorescence (total group) (a.u.)	2.74 (0.7)

Values are expressed as mean (SD). *Median and Interquartile range.

Table 2 shows the mean baseline skin AF of the 881 survivors subdivided in groups with continued absence or presence or the development of microvascular complications at follow-up. During a median follow-up period of 3.1 years, 61 patients (7.0%) developed retinopathy; their baseline skin AF did not differ from skin AF levels of patients who did not show or already had retinopathy at baseline. However, skin AF was higher in the patient groups who developed neuropathy or (micro)albuminuria compared to those without these complications. At follow-up newly developed neuropathy was diagnosed in 7.5% and newly developed (micro)albuminuria was found in 10.1%; 12.5% of the population developed at least one microvascular complication. Skin AF at baseline was also significantly higher in the patient groups who developed any microvascular complication or who already had a microvascular complication at baseline, compared to those patients who did not develop any microvascular disease at all.

Table 2. Mean skin AF (SD) at baseline and mean differences between groups

Microvascular complication		A= t_0 : absent t_{fu}: absent	B= t_0 : absent t_{fu}: present	C= t_0 : present t_{fu} : present		B vs. A	C vs. A	C vs. B
Retinopathy								
	AF	2.69 (0.73)	2.88 (0.74)	2.91 (0.72)	**ΔAF**	0.20	0.22	0.02
	n	647	61	169	**95% CI**	-0.04-0.43	0.07-0.37	-0.24-0.29
					p	0.14	0.002	1.00
Neuropathy								
	AF	2.67 (0.72)	2.93 (0.75)	2.88 (0.75)	**ΔAF**	0.26	0.21	-0.05
	n	596	66	215	**95% CI**	0.03-0.49	0.07-0.35	-0.29-0.20
					p	0.019	0.001	1.00
(Micro)albuminuria								
	AF	2.62 (0.68)	2.91 (0.67)	2.97 (0.83)	**ΔAF**	0.28	0.34	0.06
	n	570	87	207	**95% CI**	0.09-0.48	0.20-0.48	-0.16-0.28
					p	0.002	<0.001	1.00
Any								
	AF	2.52 (0.69)	2.86 (0.66)	2.88 (0.75)	**ΔAF**	0.34	0.36	0.01
	n	322	109	441	**95% CI**	0.15-0.53	0.23-0.48	-0.17-0.20
					p	<0.001	<0.001	1.00

Data are means (SD) of skin AF in arbitrary units within the group, and mean differences (ΔAF) between groups calculated with ANOVA (95% Confidence interval) with Bonferroni correction; t_0, baseline; t_{fu}, follow-up

Multinominal logistic regression analysis showed that skin AF was a strong predictor of the development of the aggregate of microvascular complications (OR 2.05 [1.51-2.80], p<0.001. Skin AF was significantly associated with the development of retinopathy (OR 1.42 [1.01-1.99], p=0.042), neuropathy (OR 1.59 [1.15-2.19], p=0.005), and (micro)albuminuria (OR 1.73 [1.28-2.34], p<0.001). After correction for the confounding risk factors, baseline skin AF still appeared to be significantly associated with the development of these endpoints, except for retinopathy (OR 1.21 [0.83-1.74], p=0.32), *Table 3*. Diabetes duration at baseline was the only significant independent variable for the development of retinopathy in this multivariate analysis (OR 1.10 [1.06-1.15], p<0.001). Surviving smokers developed less often neuropathy compared to non-smokers. In the non-surviving group (86 patients) 70% had a microvascular complication at baseline; there were 23 non-surviving smokers. Seventy percent of the non-surviving smokers already had a microvascular complication at

baseline and 13% of the non-surviving smokers developed a microvascular complication before they died.

When baseline skin AF levels are categorized in subgroups of practically feasible levels of skin AF (3 categories in rounded tertiles: skin AF < 2.35 a.u.; 2.35 ≤ skin AF < 3.00 a.u.; skin AF ≥ 3.00 a.u.), patients in the category skin AF ≥3.00 a.u do have a higher chance to develop a microvascular complication compared to patients with a lower skin AF level (*Table 4*).

Table 3. Variables related to the development of microvascular complications in type 2 diabetes mellitus by multinominal logistic regression analysis

	Any microvascular complication		Retinopathy		Neuropathy		(Micro)albuminuria	
Variables	*p value*	OR (95%CI)	*p value*	OR (95% CI)	*p value*	OR 95% CI	*p value*	OR 95% CI
Skin AF	<0.001	2.02 (1.45-2.81)	0.32	1.21 (0.83-1.74)	0.026	1.50 (1.05-2.14)	<0.001	1.88 (1.36-2.61)
Gender	0.02	0.55 (0.33-0.90)	0.91	0.97 (0.53-1.75)	0.78	1.09 (0.61-1.93)	0.001	0.42 (0.25-0.71)
HbA1c	0.004	1.30 (1.09-1.55)	0.13	1.18 (0.95-1.45)	0.87	1.02 (0.82-1.26)	0.034	1.21 (1.01-1.44)
Diabetes duration	0.66	1.01 (0.96-1.06)	<0.001	1.10 (1.06-1.15)	0.032	1.04 (1.00-1.08)	0.04	0.95 (0.90-0.997)
Smoking	0.07	0.56 (0.29-1.05)	0.09	0.48 (0.21-1.11)	0.011	0.29 (0.11-0.75)	0.96	1.02 (0.56-1.85)
Systolic BP	0.43	1.01 (0.99-1.02)	0.39	1.01 (0.99-1.02)	0.49	1.01 (0.99-1.02)	0.18	1.01 (0.996-1.02)
LDL cholesterol	0.48	1.09 (0.85-1.40)	0.66	0.93 (0.69-1.27)	0.35	0.87 (0.64-1.17)	0.30	1.15 (0.89-1.49)
HDL cholesterol	0.26	0.62 (0.27-1.43)	0.36	0.63 (0.23-1.70)	0.081	0.41 (0.15-1.12)	0.40	0.38 (0.15-0.96)
Triglycerides	0.54	0.94 (0.78-1.14)	0.41	0.91 (0.72-1.15)	0.85	0.98 (0.79-1.22)	0.19	0.87 (0.71-1.07)
BMI	0.27	1.03 (0.98-1.08)	0.33	1.03 (0.97-1.09)	0.56	0.98 (0.93-1.04)	0.39	1.02 (0.97-1.08)

Table 4. Newly developed microvascular complications subdivided in 3 skin AF-groups

n	*Microvascular complication*	*Skin AF <2.35 a.u.*	*2.35 ≤ Skin AF < 3.00 a.u.*	*Skin AF ≥ 3.00 a.u*
708	Retinopathy	15/241 (6.2)	18/251 (7.2)	28/216 (13.0)
662	Neuropathy	11/219 (5.0)	27/247 (10.9)	28/196 (14.3)
65	(Micro)albuminuria	18/225 (8.0)	31/253 (12.3)	38/179 (21.2)
431	Any	23/161 (14.3)	41/167 (24.6)	45/103 (43.7)

Number (%) of newly developed microvascular complications of subgroups; *n* is number of patients who did not have the complication at baseline

DISCUSSION

Our study provides the first evidence that skin AF is an independent predictor of the development of microvascular complications in a well-controlled type 2 diabetes population. Separately, this also holds for the development of neuropathy and (micro)albuminuria (and in univariate analysis for retinopathy). This noninvasive marker of tissue AGE accumulation may reflect the deleterious effects of long-term glycemic and oxidative stress. It was recently shown that skin AF is a predictor of 5-year coronary heart disease and mortality in diabetes [12]. The present study showed that skin AF also has a predictive value for the development of microvascular complications, which in the analysis of this study is superior to that of many other commonly used risk predictors like diabetes duration and HbA1c in type 2 diabetes. This conclusion is applicable for primary care type 2 diabetes patients, treated according to current standards, which is the large majority of type 2 diabetes patients in the Netherlands.

The DCCT/EDIC substudy already showed the predictive value for skin AGE levels obtained from skin biopsies for the progression of microvascular complications in patients with type 1 diabetes [8]. Our study population consisted of type 2 diabetes patients, with skin AGE level assessment by means of a noninvasive, rapid method. Another difference is that the DCCT-substudy investigated the development as well as the progression of microvascular complications. The limited follow-up period, the low rate of clearly classifiable progression of the microvascular complications, especially retinopathy, and the confounding role of introduced medication made us decide to restrict our study to the evaluation of the development of microvascular complications and not to address progression of these diabetic complications.

In retinopathy, skin AF turned out to have no prognostic value in the multivariate analysis. Possible explanations are the short follow-up period and the smaller amount of patients who developed retinopathy compared to the development of the other complications. Moreover the different pathophysiologic mechanisms of microvascular damage in the different organs (retina, kidneys and neurons) could play a role in the differences in incidence rates of outcomes. In particular, the pathobiology of

retinopathy might be different from those in the kidney and neurologic system due to a different role of vascular endothelial growth factor (VEGF) as a possible mediator for proliferation [20].

(Micro)albuminuria is an early clinical sign of diabetic nephropathy; when left untreated it predicts a high risk for the development of progressive renal damage which will eventually may lead to end stage renal disease. Progressive renal disease is also associated with a vastly increased cardiovascular risk. This study defined (micro)albuminuria as a sign of microvascular complication with the intention to reflect early stages of diabetic nephropathy.

In the predictive analyses the non-surviving patients were excluded from the analyses. These non-survivors had markedly increased skin AF values, but they also had a very high prevalence of microvascular complications at baseline (70%), so this does not reduce the strength of the relation between skin AF and microvascular complications at all.

Ethnicity is one of the mentioned UKPDS confounding risk factors for the development of microvascular disease. Because of the limitation of measuring skin AF in dark skin types, with the prototype of the AGE reader used in the present study, people with dark skin had to be excluded. Over 95% of the participants were Caucasian, therefore ethnicity was not taken into account in the analyses. Further developments of the AGE reader may hopefully enable measurements in dark skin type in future investigations.

Lutgers et al. previously described the other limitations of the AFR as a marker of tissue AGE accumulation: non-fluorescent AGEs will not be measured with the AFR, and other tissue components which fluoresce in the same range of wavelength might be confounders [11].

In conclusion, our study confirms skin autofluorescence as a helpful clinical method to identify type 2 diabetes patients who are at risk for (developing) any microvascular complication, neuropathy and (micro)albuminuria. Further investigation with longer follow-up needs to be done to assess whether or not skin autofluorescence is a factor in the development of diabetic retinopathy, and to assess the relationship of skin AF and

the progression of microvascular complications. Its non-invasive and time-saving application makes the AFR an easy clinical tool useful in the out-patient clinic in the risk assessment as well as for monitoring changes in accumulation of tissue AGEs reflecting long term glycemic stress.

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CHAPTER 8

Summary and general discussion

Summary

Advanced glycation endproducts (AGEs) are a heterogeneous group of compounds formed by glycation and oxidation of proteins. AGEs accumulate on long-lived proteins like collagen. This results in increased cross-linking and causes changes of structure and function of the proteins with consequently damaging effects on tissue. Besides, AGEs bind to receptors, especially the receptor for advanced glycation endproducts (RAGE) [1]. This AGE-RAGE complex induces intracellular transduction mechanisms and cellular activation which can also result in tissue damage. Because of the stability of AGEs, they accumulate slowly over a person's lifespan. AGE accumulation is accelerated in conditions with increased supply of substrates (carbonyl stress), increased oxidative stress, decreased clearance of AGE moieties, or a combination. Diabetes mellitus, rheumatic diseases and renal failure are examples of diseases with accelerated AGE accumulation. The altered structure and function of proteins by crosslinked AGEs play a pathogenetic role in the complications of these diseases.

In diabetes mellitus, AGE accumulation in skin collagen is related both with cumulative glycemic and oxidative stress, and with the presence of long-term complications [2,3]. Tissue AGE assessments previously required invasive sampling. AGEs linked to collagen have a characteristic fluorescence spectrum at 440 nm upon excitation at 370 nm. Classically, this ‘Collagen Linked Fluorescence’ (CLF) has been used to quantify tissue AGE accumulation in extracts from homogenates of skin biopsies [2]. More recently developed biochemical and immunochemical assays measuring fluorescent en non-fluorescent AGEs can be applied to skin or blood samples [4]. These techniques are unfortunately time-consuming and expensive. A further concern of serum or plasma AGE assays is that they have poor relation with tissue contents of AGEs and initially low reproducibility.

Skin autofluorescence (AF) represents the fluorescence of skin tissue fluorophores which was earlier serendipitously discovered to be increased in patients with diabetes [5]. Combining that observation with the existing literature about fluorescence of specific AGEs and CLF, it was recognized as a potential non-invasive

measure for tissue AGE-accumulation. Considering this, since 1996 the Autofluorescence Reader (AFR) was developed to provide a marker representative for tissue AGE-accumulation to be easily applicable in a clinical setting for predicting diabetes related complications.

The validation study of the AFR showed good relations between skin autofluorescence and fluorescent and non-fluorescent AGEs in skin-biopsies, and also good reproducibility [6]. In a later study, it was shown that skin AF appeared to be a predictor for cardiovascular complications in diabetes patients [7]. The study population was, however, a small, mixed group of type 1 and type 2 diabetes patients.

In the present thesis, we studied prospectively in a large type 2 diabetes population (~1000), whether skin AF is a risk indicator for chronic diabetes complications and cardiovascular morbidity and mortality. We furthermore evaluated in different groups of diabetes patients (type 1, type 2, both with and without complications), whether the excitation spectrum of the AFR needed to be further optimized to detect possible differences in fluorescence characteristics in these different populations.

Chapter 1 shortly outlines background information about the skin and its biochemical composition, the development of the Autofluorescence Reader (AFR), and the calculation of skin AF. Since skin pigmentation may influence autofluorescence by light absorption, skin AF was calculated by dividing the average emitted light intensity per nanometer in the range 420–600 nm by the average excitated light intensity per nanometer in the range 300–420 nm.

Chapter 2 demonstrates the validity of a broad excitation wavelength range, such as applied in the AFR. In this chapter, an adapted version of the AFR was used: a monochromator, allowing to study the effects of small wavelength ranges of the excitation light, was connected to a measuring component that was similar to the AFR. With this instrument, excitation-emission maps were obtained in vivo from the skin of selected groups of type 1 and type 2 diabetes patients with and without complications, and in healthy controls. Consistent with our expectations, AF was increased in both type 1 and type 2 diabetes patients with complications compared to the diabetes

patients without complications. We found however no specific excitation wavelengths in the range 355 - 405 nm that would yield an increased distinction between diabetes patients with or without chronic complications.

We furthermore evaluated whether specific excitation wavelengths could induce different fluorescence peaks corresponding with different fluorophores/AGEs. We hypothesized a possible difference of excitation-emission maps in type 1 and type 2 diabetes, as these diseases with a different metabolic condition might generate different AGEs. We did not find however differences between these groups that depend on excitation wavelength and concluded that the involved fluorophores do not necessitate the use of a specific excitation wavelength between 355 and 405 nm.

Different mechanisms of AGE formation that have been described over the last decades were summarised in **Chapter 3**. Besides the classical view involving the Maillard reaction, the role of oxidative and carbonyl stress, as well as overproduction of superoxide by the electron transport chain in mitochondria in endogenous AGE formation are demonstrated. These different pathways of AGE formation imply multiple substrates for AGE formation. This emphasises that accelerated AGE formation is not unique for diabetes/hyperglycaemia, but that AGE accumulation can occur in many more conditions characterised by increased supply of substrates and oxidative stress, for example in rheumatic diseases, ischemic heart disease, and renal dysfunction. This also suggests that in a metabolic disease like type 2 diabetes with concurrent problems like dyslipidemia and nephropathy, AGE accumulation could be an accurate measure of cumulative metabolic stress and is, therefore, supposed to be related to chronic complications of diabetes. Previous studies in fact showed this relation [8-11].

The developments of the different strategies in reducing AGE accumulation were also reviewed in this chapter, e.g. AGE formation inhibitors (aminoguanidine, pyridoxamine, benfotiamine and ACE-inhibitors) and AGE breakers (ALT-711). ALT-711 and benfotiamine are currently being investigated in phase II studies. A novel AGE-breaker, C36, also a thiazolium-based AGE-breaker like ALT-711, claims less mutagenesis. In diabetic rats, C36 was demonstrated an effective AGE-breaker by

showing beneficial effects on the cardiovascular system [12]. Unfortunately, since the first studies on aminoguanidine in the late 1980s, the development of specific AGE-inhibitors or AGE-breakers did not result in clinically available drugs yet.

The role of AF in diabetes and its relation to clinically used variables including the presence of diabetes complications in a cross-sectional study was described in **Chapter 4**. This study involves an analysis of the baseline data of the Zwolle type 2 diabetes study cohort, as well as a non-diabetic control group. Skin autofluorescence was significantly higher in type 2 diabetic patients compared with control subjects in each age category, which was in agreement with the results of Meerwaldt et al [6]. Multiple regression analysis showed significant correlation of skin autofluorescence with age, sex, diabetes duration, BMI, smoking, HbA1c, plasma creatinine, HDL cholesterol, and albumin-to-creatinine ratio. HbA1c had a small independent contribution to autofluorescence. Skin autofluorescence was associated with a graded increase in the presence and severity of diabetes-related complications.

In **Chapter 5** we studied in a follow-up study whether skin autofluorescence has an additional value to the UKPDS risk engine. The UKPDS is a landmark randomized controlled trial conducted in the ~1990s in individuals newly diagnosed with type 2 diabetes. It showed that both intensive treatment of blood glucose and blood pressure in diabetes can lower the incidence of diabetes-related complications [13,14]. A prediction model for fatal and non-fatal cardiovascular disease was extracted from these data, resulting in the UKPDS risk engine [15]. They showed that the addition of HbA1c to traditional risk factors improved risk prediction in T2DM-patients. Although this risk engine is the first calculator including HbA1c and consists of 10 different items, it still has a limited positive and negative predicted value for CV-events in T2DM [16]. In this chapter we studied whether skin autofluorescence has an additional value to this UKPDS risk engine. In a multivariate Cox regression analysis with all items of the UKPDS risk engine, skin autofluorescence was a significant predictor of fatal and non-fatal CV-events, together with age, diabetes duration and gender. A second multivariate analysis including a history of microvascular complications,

macrovascular complications, a UKPDS risk score >10% and skin autofluorescence, all were significant predictors for non-fatal CV-events and mortality except a history of microvascular complications. Furthermore, when a skin AF cut-off value at the median is used for risk reclassification of patients with a UKPDS risk score of <10%, cardiovascular risk of 55 of the 203 of the patients (~25%) would be reclassified from low risk to high risk. Finally, skin AF was able to identify a patient group with a more increased event rate within the group of patients with a high risk for fatal cardiovascular event of more than 10% within the next 10 years. These findings could have treatment consequences with intensifying all aspects of individual diabetes management aiming for reduction of the development or progression of complications.

The effects of improvements in diabetes care, since the results of the UKPDS were published at the end of the 1990s, has become obvious in a major improvement of life expectancy, as shown in **Chapter 6**. Until recently, most diabetes papers started in their introduction with the statement cardiovascular morbidity and mortality is increased in people with diabetes. Since 2006, the first papers reported the reduction of mortality-rate in diabetes. In a five-year follow-up study of our type 2 diabetes cohort, we found an equal life expectancy of the T2DM subjects compared to the general Dutch population. In this analysis, a history of cardiovascular disease and albuminuria were significant risk factors for early mortality (death before end of life expectancy was accomplished).

Previous chapters have shown the relationship between skin AF and macrovascular complications, as well as its predictive value for total and cardiovascular mortality in type 2 diabetes mellitus. In **Chapter 7** we investigated the predictive value of skin AF for the development of microvascular complications in the Zwolle type 2 diabetes study cohort. The Diabetes Control and Complications Trial (DCCT), the landmark study in type 1 diabetes published in 1993, showed that intensive treatment delays the onset and slows the progression of microvascular complications compared to conventional diabetes therapy [17]. A substudy of the DCCT, conducted by the DCCT skin collagen ancillary study group, evaluated the effects of long term intensive glycemic control on indicators of skin collagen glycation

(furosine), glycoxidation and AGE-formation (pentosidine and carboxymethyllysine [CML]), and crosslinking (acid and pepsin solubility). They showed lower levels of AGEs in skin collagen in the intensive treatment group compared to the conventional treatment group [10]. It was also shown that skin AGE levels predicted the risk of development or progression of microvascular disease in type 1 diabetes mellitus, even after adjustment for HbA1c [10,11]. In our study in type 2 diabetes, skin AF at baseline was significantly higher in patients who developed any microvascular complication, or neuropathy or (micro)albuminuria alone, after a mean follow-up period of ~3 years. Multivariate analyses showed that skin AF remained as a predictor for the development of any microvascular complication along with HbA1c; for the development of neuropathy along with smoking, and for the development of (micro)albuminuria together with gender, HbA1c and diabetes duration. Skin AF did not have predictive value for the development of retinopathy separately.

Discussion, future perspectives and conclusion

It is concluded that skin autofluorescence represents cumulative metabolic stress and is therefore successful in predicting complications in type 2 diabetes mellitus. This was shown in this thesis for microvascular, macrovascular complications and for mortality. Moreover, skin AF was demonstrated to be superior to HbA1c and other conventional risk factors (like smoking, blood pressure and blood lipids). It furthermore provides clinically relevant additional information to the UKPDS risk engine in the prediction of cardiovascular events and mortality. Finally, we concluded that there was no difference in fluorescence spectra using the broad excitation spectrum of the Autofluorescence Reader/AGE-Reader or a variable specific excitation wavelength or set of wavelengths (between 355-405 nm).

The limited role of HbA1c compared to skin autofluorescence or AGE-accumulation in predicting complications in type 2 diabetes emphasises the importance of ‘metabolic memory’ rather than ‘glycemic memory’. Metabolic memory also includes AGEs formed during oxidative stress or other pathways of AGE formation using other substrates than glucose. Especially in a condition like type 2 diabetes where

besides hyperglycaemia multiple metabolic disturbances are present, measurement of AGEs could be more representative for cumulative metabolic stress compared to HbA1c. A second restriction of HbA1c is that post-prandial hyperglycaemia is not fully included, with consequently underestimation of cumulative metabolic stress. Post-prandial hyperglycaemia might be important in developing AGE-accumulation and complications. The high glucose value induces oxidative stress, and at the same time, lots of substrate are available such as blood lipids and amino-acid levels that are also increased after a meal. The short turnover time of HbA1c is the third major explanation for the restricted relation between HbA1c, AGEs and complications. HbA1c represents glycemic control of the last 8 weeks. As the half-life of skin collagen is 15 years, it is likely that skin AGE levels provide a more "long-term memory" of glycemic stress and are, therefore, better in predicting complications. This should be further explored in future studies on the impact of integrated long-term follow-up of HbA1c measurement on skin AF, and also on the development of complications.

Although it is not established whether skin autofluorscence, a technique based on fluorescent properties of certain AGEs, is a good measure for non-fluorescent AGEs, there are reasons to assume it is. Firstly, in the validation study, the correlation between skin AF and fluorescent AGEs/CLF was as good as with non-fluorescent AGEs, like CML. CML is also known as a product formed during oxidative stress or lipoxidation. Secondly, the thesis of Mulder (2007) showed multiple positive relations between skin AF and markers of oxidative stress [18]. Likewise, relations of skin AF with CRP and indirect oxidative stress markers (such as vitamin C) were also reported by Meerwaldt and Hartog [19,20]. Finally, the additional value of skin AF to the UKPDS risk engine in prediction of complications also suggests that this risk engine (which includes the traditional risk factors) does not fully incorporate 'metabolic memory' relevant for cardiovascular prognosis. The additional prognostic value of accumulated oxidative stress might be better covered by skin AF.

Results of ongoing studies with follow-up measurements of skin AF are needed. Follow-up measurements of skin AF will provide information on the rate of AGE-accumulation. The use of repeated skin AF measurements in intervention studies with

e.g. benfotiamine and ALT-711 may also reveal which interventions are able to decelerate the AGE accumulation process to finally prevent complications in diabetes. Follow-up measurements may also answer the question whether a single AF measurement is sufficient for an individual patient to predict future complications, or that assessing the AF progression rate by multiple longitudinal measurements is better. Until now, one AF measurement has proven to be a good predictor for diabetes complications.

The broad excitation wavelength range, such as applied in the AFR, is adequate for measuring the fluorescence of the fluorophores in different types and stages of diabetes. The current use of a relatively broad emission wavelength range for fluorescence does not have to be changed either. So no adjustments of the tool have to be made for applying it in all the studied groups. Technically, this implies that next generations of the currently used AGE-Reader may be developed with relatively simple light sources and less expensive spectrometers, which would make the tool less costly facilitating extended use in for instance primary care or even for screening application. A recent paper showed a higher sensitivity for detection of 'impaired glucose tolerance' or type 2 diabetes compared to the OGTT or a single HbA1c measurement [21]. Although more and larger population studies are necessary for implementing skin AF as a screening tool for (pre)diabetes, it would be an important improvement if increased cardiovascular risk could be detected earlier to decelerate the AGE accumulation rate in an earlier state preventing cardiovascular diseases.

There was no significant seasonal variation of skin AF in the exclusively Caucasian study population. One important limitation of the used AFR has been its unreliability in people with dark skin. Recent improvements of the AGE-reader now allow to extend the application on non-Caucasian subjects. In these subjects, validation studies to assess the relation of skin AF with diabetes complications are now ongoing.

There are no specific AGE-inhibitors or AGE-breakers clinically available yet, despite efforts to develop them since the 1980s. The cause of this disappointing development track could be toxicity problems, lack of efficacy or monitoring issues to detect decrease in AGE-levels. For aminoguanidine and pyridoxamine problems with

toxicity have indeed occurred, while in some studies efficacy has been found to limited. A general problem with AGE-breakers might be that breaking AGEs results in AGE degradation products which can be highly reactive and form new AGEs again contributing to low clinical efficacy. In vivo monitoring of effectiveness of interventions may also be difficult as mostly blood AGE levels are used which can even increase when tissue AGEs have degradated and entered into the circulation. Skin autofluorescence is expected to reflect tissue AGE accumulation and might therefore be better in monitoring a decrease in total body AGEs induced by AGE-breakers. Application of the AGE-Reader for monitoring therapeutic interventions has started in several ongoing studies and may so contribute to the development of a clinically available AGE-breaker.

Since metabolic damage due to oxidative stress and inflammation can result in 'accelerated aging' and its complications, the application of skin autofluorescence could be extended to other fields of medicine. Recently, increased skin autofluorescence has been reported in rheumatoid arthritis and in systemic autoimmune diseases, but other populations of interest include patients with amyotrophic lateral sclerosis, or identification of accelerated cardiovascular disease after chemotherapy [22,23]. Studies in such patient cohorts are in progress. Data of these studies are also important for general health care. Since treatment of diseases like acute myocardial infarction, cancer or post-transplantation immunosuppressive therapy had been improved, mortality in these patient groups are dramatically decreased over the past decades. Incidence of chronic diseases, like heart failure has, however, enormously increased with subsequently rise of medical costs. Follow-up skin autofluorescence measurements may provide insight in the future course of 'chronic complications' by therapy induced accelerated aging, and consequently insight to future expenditure and/or improvement of medical treatment strategies.

In conclusion, skin autofluorescence may become a generally used clinical tool for estimating cardiovascular risk in conditions with increased oxidative stress and supply of substrates, especially in diabetes mellitus. Monitoring interventions might

become a second application of the skin autofluorescence measurement aiming for further reduction of the incidence of diabetes complications by stimulating the development of AGE-inhibitors or AGE-breakers.

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CHAPTER 9

Samenvatting

Advanced glycation endproducts (AGEs) ontstaan via chemische reacties waarbij er versuikering en oxidatie optreedt van o.a. eiwitten en vetten, waarbij vaak een onomkeerbare verbinding ontstaat met eiwitten, zoals collageen. Bij ieder mens stapelen AGEs gedurende het leven, maar bij iemand met diabetes mellitus gebeurt dit versneld. AGEs hebben een belangrijke rol bij het ontstaan van chronische complicaties van diabetes en atherosclerose.

Tot recent werden weefsel-AGEs gemeten in huidbiopten. Dat is een belastende methode, ongeschikt voor toepassing op grote schaal, of voor herhaalde metingen. Het is vanuit de literatuur bekend dat sommige AGEs fluoresceren. Enkele jaren geleden is door de Biomedical Engineering van de Rijksuniversiteit Groningen en de afdeling Interne Geneeskunde van het UMCG de Autofluorescence reader (AFR) ontwikkeld, dat gebruikmakend van fluorescentieprincipes, niet-invasief autofluorescentie van de huid meet. De AFR is een draagbaar apparaat, waarin een UV-lamp (excitatie, golflengte 355-405 nm), waarop de onderarm kan worden gelegd. Een spectrometer analyseert de fluorescentie die de huid uitzendt (emissie, golflengte 420-600 nm), waarna de computer de meetgegevens binnenkrijgt en verwerkt tot een getal (=ratio van de gemiddelde emissie en de gemiddelde excitatie). Uit de validatiestudie bleek dat autofluorescentie een goede maat was voor weefsel-AGEs.

Inmiddels zijn er meerdere studies met de AFR verricht. Hierin werd o.a gezien dat autofluorescentie bij patiënten met diabetes, nierinsufficiëntie en tijdens een acuut hartinfarct hoger is ten opzichte van gezonde proefpersonen van dezelfde leeftijd. Verder bleek autofluorescentie in een kleine gemengde groep patiënten met type 1 en type 2 diabetes voorspellend te zijn voor cardiovasculaire complicaties. Inmiddels is het prototype AFR verder ontwikkeld tot de AGE-Reader.

De doelen van de studies beschreven in dit proefschrift zijn als volgt. Ten eerste wilden we nagaan of verschillende aandoeningen (diabetes type 1 en type 2, met en zonder diabetische complicaties) ook tot verschillende fluorescentie spectra leiden. Met andere woorden, hebben mensen met type 1 diabetes andere fluorescerende stoffen/AGEs in hun huid t.o.v mensen met type 2 diabetes. En, is er verschil in AGEs bij diabetes patiënten met en zonder complicaties. Ten tweede hebben we in een

prospectief type 2 diabetes cohort (973 patiënten) uit de regio Zwolle uitgezocht wat de relatie is tussen autofluorescentie van de huid en bekende klinische variabelen bij diabetes als HbA1c (= geglycosyleerd hemoglobine) en reeds aanwezige micro- en macrovasculaire complicaties, en of autofluorescentie ook een voorspeller is voor nieuwe complicaties en overlijden. Alle studies beschreven in de hoofdstukken vanaf hoofdstuk 4 zijn uitgevoerd bij hetzelfde type 2 diabetes cohort uit de regio Zwolle.

Hoofdstuk 2 beschrijft de studie waarin het eerste onderzoeksdoel is uitgezocht. Bij 5 verschillende groepen van ieder ongeveer 20 personen met een leeftijd tussen 35 en 50 jaar (controle groep, type 1 diabetes patiënten zonder complicaties, type 1 diabetes patiënten met complicaties, type 2 diabetes patiënten zonder complicaties, type 2 diabetes patiënten met complicaties) werd autofluorescentie van de huid gemeten. Dit werd gedaan met een Excitatie-emissie scanner (EEMs), waarbij er niet zoals bij de normale meetopstelling (AFR) met licht van 1 breder golflengtebereik (355-405 nm) wordt gewerkt, maar smallere golflengtegebieden (à 5 nm) binnen het genoemde bereik op de huid werd geschenen. De hoeveelheid fluorescentie (emissie) en de vorm van het fluorescentiespectrum, geïnduceerd door de diverse smallere golflengtegebieden (excitatie), werd vergeleken tussen de verschillende groepen. De hoeveelheid autofluorescentie was verhoogd bij beide diabetesgroepen met complicaties t.o.v de diabetesgroepen zonder complicaties. Er was echter geen verschil in fluorescentiekarakteristieken tussen controle personen, type 1 en type 2 diabetes patiënten. Ook was er geen verschil in aanwezige fluorophoren in de huid bij aan- of afwezigheid van diabetes gerelateerde complicaties. Hieruit kan worden geconcludeerd dat het brede excitatie golflengtegebied, zoals gebruikt bij de AFR voldoet en niet verder hoeft worden aangepast om autofluorescentie van de huid te meten bij diabetes patiënten.

Hoofdstuk 3 is een bespreking van de literatuur die bekend is rondom de verschillende mechanismen die leiden tot vorming van AGEs, en de rol van AGEs bij het ontstaan van de complicaties van diabetes zoals retinopathie, neuropathie en nefropathie. Ook wordt beschreven dat AGEs een rol hebben bij normale

verouderingsprocessen (cataract, atherosclerose), nierziekten en de ziekte van Alzheimer. Tot slot komt in dit hoofdstuk aan de orde de verschillende geneesmiddelen die sinds enkele jaren in ontwikkeling zijn om stapeling van AGEs te kunnen remmen, en daarmee hopelijk ontwikkeling van complicaties vertragen of voorkomen. Voorbeelden van zogenaamde AGE-inhibitors zijn aminoguanidine en pyridoxamine, een voorbeeld van een AGE-breaker is ALT-711 (alagebrium).

In **hoofdstuk 4** wordt bij het Zwolse type 2 diabetes cohort (n=973) een cross-sectionele analyse uitgevoerd van de basismeetgegevens die tussen 2001 en 2002 zijn verzameld. Autofluorescentie van de huid was gerelateerd aan leeftijd, geslacht, diabetesduur, BMI, roken, HbA1c, HDL-cholesterol en albuminurie. Verder bleek hoe meer complicaties er bij de patiënt reeds aanwezig waren (microvasculair, macrovasculair of beide), hoe hoger de autofluorescentie waarde.

Voor de risico-inschatting op het ontwikkelen van een fataal of niet-fataal cardiovasculair incident bij type 2 diabetes patiënten is sinds enkele jaren de UKPDS risicoscore beschikbaar. Deze risicoscore komt voort uit de United Kingdom Prospective Diabetes Study, waarbij >4000 nieuw ontdekte type 2 diabetes patiënten werden behandeld volgens een conservatief schema of volgens een intensief behandelschema. In **hoofdstuk 5** werd geanalyseerd of autofluorescentie van de huid van toegevoegde waarde is aan de UKPDS risicoscore, bij het voorspellen van cardiovasculaire incidenten en dood. De resultaten van deze studie toonde dat binnen de groep patiënten die al een hoog risico (10-jaars risico op fataal CV-incident >10% met UKPDS risico score) heeft op cardiovasculaire complicaties, autofluorescentie een subgroep kan identificeren met een nog sterker verhoogd risico. Verder leidt toevoeging van autofluorescentie tot reclassificatie van 27% van de patiënten uit de laagrisico patiënten (UKPDS risicoscore <10%) naar een hoogrisico groep (=autofluorescentie groter dan de mediaan). Het reeds hebben van een macrovasculaire complicatie, een UKPDS risico score >10% en een autofluorescentie waarde boven de mediaan, zijn allen onafhankelijke voorspellers voor nieuwe cardiovasculaire incidenten en dood.

Hoofdstuk 6 laat zien dat de levensverwachting van dit type 2 diabetes cohort vergelijkbaar is met de levensverwachting van de Nederlandse bevolking. In het cohort bevinden zich type 2 diabetes patiënten uit de eerstelijns gezondheidszorg (=uit de huisartspraktijk), die in het kader van 'transmurale zorg' jaarlijks door een diabetesverpleegkundige gescreend worden op complicaties, waarna de huisarts aan de hand van de bevindingen van de diabetesverpleegkundige en laboratoriumuitslagen een behandeladvies krijgt van een internist. Het halen van een normale levensverwachting bij mensen met type 2 diabetes is dus mogelijk, indien de patiënt zich in een dergelijk zorgprogramma bevindt. Binnen het normale levensverwachtingprofiel voor de hele groep heeft een individuele type 2 diabetes patiënt met een macrovasculaire complicatie in de voorgeschiedenis of albuminurie wel een toegenomen risico om vervroegd te overlijden.

In **hoofdstuk 7** werd geanalyseerd of autofluorescentie een voorspeller is voor microvasculaire complicaties, zoals retinopathie, neuropathie en nefropathie. Baseline incidentie van microvasculaire complicaties werd vergeleken met nieuw opgetreden microvasculaire complicaties, na een follow-up van ~3 jaar. Autofluorescentie bleek de sterkste voorspeller te zijn voor alle nieuwe microvasculaire complicaties samen, ten opzichte van HbA1c en diabetesduur. Bloeddruk en lipiden waren niet significant. Voor de microvasculaire complicaties afzonderlijk, blijkt autofluorescentie alleen voorspellend voor neuropathie en microalbuminurie en niet voor retinopathie. Voor retinopathie was diabetes duur alleen voorspellend.

Concluderend, autofluorescentie van de huid is in een grote groep type 2 diabetes patiënten een voorspeller voor de incidentie van microvasculaire, macrovasculaire complicaties en dood. Het is van aanvullende waarde aan de UKPDS risico-inschatting op het optreden van fatale en niet-fatale cardiovasculaire incidenten binnen 10 jaar. Bij het uitrekenen van een dergelijke 10-jaars risico score, zullen patiënten al gauw hoger dan 10% scoren. In de studie populatie uit dit proefschrift scoorde 79% boven de 10%. Toevoegen van autofluorescentie kan dan binnen deze hoogrisico groep, individuele patiënten identificeren met een extra hoog risico. Dit kan er toe leiden dat deze extra

hoogrisico patiënten nog strikter volgens de richtlijnen worden behandeld of bv. laagdrempeliger worden doorgestuurd voor cardiologische evaluatie.

De UKPDS risico score kan ook foutief te laag uitvallen, als in het model behandelde waardes van HbA1c, bloeddruk en lipiden wordt ingevuld. Het cardiovasculair risico van de patiënt wordt dan onderschat. Als de UKPDS score laag uitvalt en de autofluorescentie waarde wel hoog is, dient patiënt gereclassificeerd te worden naar een hoogrisico groep. Aangezien uit dit proefschrift blijkt dat autofluorescentie een maat is voor vastgestelde vasculaire schade maar ook voor preklinische vasculaire schade, kan autofluorescentie dus van toegevoegde waarde zijn. Ook voor risico-inschatting bij patiënten, waarbij type 2 diabetes recent is vastgesteld kan een autofluorescentie meting aanvullende informatie geven, omdat het vaak onduidelijk is hoelang de ziekte bestaat en wat de risico's op korte of langere termijn zijn voor het ontwikkelen van diabetische complicaties.

Follow-up studies met vervolgmetingen van autofluorescentie over de tijd (jaren) zijn gaande en moeten aantonen wat de progressie snelheid van autofluorescentie is, welke factoren deze progressie snelheid kunnen vertragen en of een eenmalige autofluorescentie meting genoeg is om de risico-inschatting te maken ten aanzien van het voorspellen van complicaties of dat meerdere autofluorescentie metingen danwel de 'autofluorescentie progression-rate' beter zijn.

Een ander mogelijk toepassingsmogelijkheid van de AFR/AGE-Reader in de toekomst, is AGE-monitoring bij interventiestudies met de in ontwikkeling zijnde "AGE-inhibitors" en "AGE-breakers". Als de AFR de uitvoering van klinische interventie trials kan vergemakkelijken, dan zou de klinische introductie van AGE-inhibitors versneld kunnen worden, met als doel een daling van microvasculaire en macrovasculaire complicaties ten gevolge van diabetes mellitus.

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One. Jan Willem, met jou wil ik blijven dansen op dit liedje

CURRICULUM VITAE

Helen Lucia Lutgers werd geboren op 25 januari 1976 in Hoogezand-Sappemeer. Ze heeft één broer. Zij volgde het atheneum op het Aletta Jacobs College in Hoogezand en behaalde in 1994 haar VWO examen. Datzelfde jaar begon zij met de studie geneeskunde aan de Rijksuniversiteit Groningen. In 1997 begon ze samen met Lyanne Hullegie met het 'Glucose sensor' onderzoeksproject bij de vakgroep Endocrinologie van de Interne Geneeskunde in het Academisch Ziekenhuis Groningen o.l.v. dr. R.P.F. Dullaart, dr. K. Hoogenberg en dr. A.J.M. Schoonen. Dit project werd een wetenschappelijke stage en resulteerde o.a in een voordracht op het Groninger Studenten Congres 1999, waarbij ze de KNMG-prijs voor 'meest maatschappelijk relevant onderzoek' won, een posterpresentatie op de EASD in Brussel en een eerste publicatie in een peer-reviewed tijdschrift.

Aansluitend startte ze haar co-schappen, die in het Academisch Ziekenhuis Groningen, Meppel en in Drachten werden gelopen. Haar keuzeco-schap deed ze in Zwolle, Isala klinieken, begeleider H.J.G. Bilo. Op 25 januari 2001 werd zij bevorderd tot arts (cum laude).

Hierna werkte zij als ANIOS bij de afdeling Longziekten/Longtransplantatie en Interne Geneeskunde in het Universitair Medisch Centrum Groningen. Vanaf oktober 2001 startte ze met het AGIKO-project 'Skin autofluorescence in diabetes mellitus'.

Vanaf oktober 2003 begon zij met de opleiding Interne Geneeskunde in de Isala klinieken te Zwolle; opleider dr. M.A. Alleman en later dr. M. van Marwijk Kooy. Vanaf januari 2007 vervolgde ze haar opleiding in het Universitair Medisch Centrum Groningen; opleider prof. dr. R.O.B. Gans. Sinds 1 oktober 2008 is zij gestart met haar aandachtsgebied Endocrinologie, opleider dr. R.P.F. Dullaart.

Helen is sinds 5 oktober 2007 getrouwd met Jan Willem Haveman.

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