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Skin autofluorescence in diabetes mellitus

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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 excited 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), glycooxidation 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 autofluorescence, 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 degraded 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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