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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Arsov, S. (2013). *Advanced glycation end-products in hemodialysis patients*. [Thesis fully internal (DIV), University of Groningen]. [s.n.].

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Stefan Arsov

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Stellingen behorende bij het proefschift

Advanced Glycation End-Products in Hemodialysis Patients

Stefan Arsov

1. Hemodialysis is a life threatening therapy for a life threatening condition. *(This thesis book)*

2. Skin autofluorescence is a mirror of dermal tissue. *(This thesis book)*

3. Skin biopsies for assessment of skin AGEs are useless. *(This thesis book)*

4. The AGE Reader is a simple tool to use, but the results are not simple. *(This thesis book)*

5. It is advisable to measure skin AGEs in hemodialysis twice a year. *(This thesis book)*

6. Slight overweight does not harm health. *(This thesis book)*

7. All you need is love. But a little chocolate now and then does not do harm.

8. My reins shall rejoice. *(Proverbs 23:16 King James Version)*

9. Beauty is only skin deep.

10. The human skin records the past as tree rings do.

11. If we open a quarrel between *past and present*, we shall find that we have lost the *future*. *(Winston S. Churchill)*

12. There are no positive or negative results; there are only correct and incorrect results.

Financial support for the publication of this thesis was kindly provided by:

Diagnoptics

DiagnOptics Technologogies B.V.

WJKI
W.J.KOLFF INSTITUTE
FOR BIOMEDICAL ENGINEERING AND MATERIALS SCIENCE

W.J. Kolff Institute

umcg

University Medical Center Groningen

RIJKSUNIVERSITEIT GRONINGEN

Advanced glycation end-products in hemodialysis patients

Proefschrift

ter verkrijging van het doctoraat in de

Medische Wetenschappen

aan de Rijksuniversiteit Groningen

op gezag van de

Rector Magnificus, dr. E. Sterken,

in het openbaar te verdedigen op
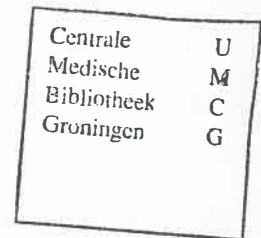
woensdag 6 november 2013

om 12:45 uur

door

Stefan Arsov

geboren op 2 mei 1980

te Skopje, R. Macedonië

Promotores: Prof. dr. G. Rakhorst

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Chapter 1

Introduction

Chronic kidney disease (CKD) is characterized by a progressive loss of renal function over a period of months or years. The progression of CKD may be so slow that symptoms do not appear until kidney function is less than one-tenth of its normal value. The first symptoms of CKD are unspecific and can vary from a loss of appetite to a feeling of general discomfort. CKD is often diagnosed during routine screening of people with high risk of renal dysfunction, such as elderly persons, patients with high blood pressure and patients with diabetes mellitus. This condition is also often identified after it manifests its complications, such as cardiovascular disease, anemia or pericarditis[1]. The Center for Disease Control and Prevention found that 16.8% of Americans, that are over 20 years of age suffer from CKD[2]. CKD is categorized according to its severity: stage 1 is the mildest stage of CKD and stage 5 the most severe. This score is based on the level of the glomerular filtration rate (GFR) that has been normalized to the body surface area. GFR is the volume of fluid filtered from the renal glomerular capillaries into the Bowman's capsule per unit of time. The normal GFR is in range of 100-130 ml/min/1.73m^2. There are several different ways to estimate GFR. A simple serum creatinine is the easiest and most widely used marker of GFR. However just serum creatinine measurement is not very precise way of estimating GFR therefore creatinine clearance is also used. Creatinine clearance is the ratio between serum creatinine and urine creatinine excretion during a defined time period usually 24 hours, the equation is given below. However the main drawback of using creatinine clearance to estimate GFR is the time duration need to do the measurement therefore sometimes just single measurement of serum creatinine is used after correction for different variables such as, age, gender and body weight[3]

$$Creatinine\ clearence = \frac{Urine\ Creatinine\ Concentration\ X\ Urine\ Flow\ (ml\ /min)}{Plasma\ Creatinine\ Concentration}$$

The two mildest stages (stages 1 and 2) in which estimated (e)GFR is still above 60 mL/minute/1.73 m^2, require additional evidence for kidney dysfunction other than a decreased eGFR. Kidney dysfunction can be demonstrated biochemically in blood, plasma or urine, histologically in renal tissue biopsies, and finally *in vivo* with imaging techniques. The other commonly used marker of kidney dysfunction beside eGFR is albuminuria. In a large prospective study it was proven that level of albuminuria was a strong predictor of cardiovascular mortality and morbidity in subjects with albuminuria[4]. Stages 3, 4 and 5 of CKD are defined by eGFR levels that are below 60, 30 or 15 mL/minute/1.73 m^2, respectively[1].

Stage 5 of CKD is also known as End-Stage Renal Disease (ESRD). The annual incidence of ESRD in the United States in the past 30 years has increased 4 fold (Figure 1). During that period the incidence of ESRD in certain population groups has changed dramatically; the incidence of ESRD in patients older than 75 increased 30 fold whereas the incidence of ESRD in patients with diabetes mellitus or hypertension increased 15 and 25 fold respectively. As

a result the total ESRD population has a high percentage of co-morbidities. Together with the cancer population ESRD has become the biggest burden on the health care system[5].
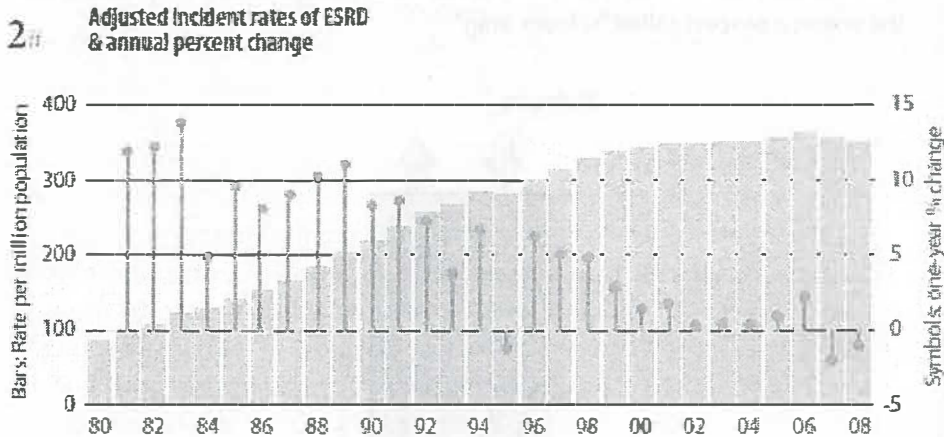
Figure1. The incidence of End-Stage Renal Disease in the United States on the left axis with bars are given the rates per million population on the right axis with ● are given one-year percent changes[4]

Subjects suffering from ESRD can only survive when they receive renal replacement therapy. There are three major types of renal replacement therapy: hemodialysis (HD), peritoneal dialysis and kidney transplantation. This thesis will focus on HD, the most frequently used method (around 70%) of renal replacement therapy.

The Principles of Hemodialysis

Dialysis is an artificial detoxification process whereby the solute composition of solution A (blood) is altered by exposing this solution to a second solution B (dialysis solution), through a semipermeable membrane. Conceptually, one can view the semipermeable membrane as a polymer sheet perforated by tiny holes or pores. Water molecules and low molecular weight solutes that are present in the two solutions can pass the membrane pores, but larger molecules such as proteins cannot. Solutes that can pass through the membrane pores are transported by two different mechanisms, diffusion and ultra-filtration:

- Diffusion is the movement of solutes as a result of random molecular motion. The diffusion is a movement of a molecule from regions of higher concentration to regions of lower concentration. Larger molecules move slower than smaller molecules. The larger molecules, even those that can fit easily through the membrane pores, will diffuse through the membrane slowly because they are moving at low velocity and colliding less frequently with the membrane.

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- Ultrafiltration occurs when water is pushed by either a hydrostatic or an osmotic force through a semipermeable membrane. In this case a concentration gradient is not necessary. The solutes that pass through the membrane are swept along with the water; a process called "solvent drag".
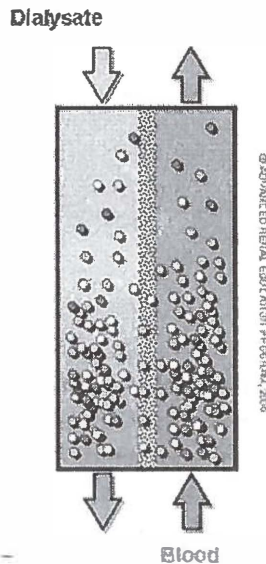
Figure 2. The working principle of Hemodialysis[5]

In clinical use, the box containing the two solutions in Figure 2 becomes the dialyzer, containing blood and the dialysis solution. The dialysis solution consists of highly purified water in which sodium, potassium, calcium, magnesium, chloride, bicarbonate, and dextrose have been administered. Low molecular weight waste products that accumulate in the uremic blood are absent in the dialysis solution. For this reason, when uremic blood is exposed to the dialysis solution via the membrane in the absence of ultrafiltration, the flux rate of these solutes from the blood to dialysate is initially much larger than the back-flux from dialysate to blood. Eventually, if the blood and dialysate were left in static contact with each other, the concentration of permeable waste products in the dialysate would become equal to that in the blood, and no further net removal of waste products would occur. Transport back and forth across the membrane would continue, but the rates of transport and back-transport would be equal. Therefore, such concentration equilibrium is prevented in practice. The concentration gradient between blood and dialysate, and thus the diffusion, is maximized firstly by continuously refilling the dialysate compartment with fresh dialysis solution and by replacing dialyzed blood with undialyzed blood. Secondly, the direction of dialysis solution flow is chosen opposite to the direction of blood flow. The purpose of this "countercurrent" flow is to maximize transport of waste products over the membrane by

maximizing the concentration difference between the blood and dialysate in all parts of the dialyzer. Ultrafiltration will of course further increase the removal of waste products[7].

Initiation of Hemodialysis in End Stage Renal Disease

In an adult ESRD patient HD is usually started when the eGFR has reached a level of approximately 10 ml/minute/1.73 m² (stage 5). However, it has been advised that the evaluation of the need for HD should begin at higher eGFR, probably somewhere around 15-20 ml/minute/1.73 m² because individuals, especially those with co-morbidities, appear to be extra vulnerable to ESRD[7].

A restriction of the use of the CKD score as an indicator for the start of the HD treatment is that it is only based on the eGFR; the CKD score does not take into consideration whether the patient has problems with CKD complications such as hyperkalemia or fluid overload. For example patients of advanced age and cognitive impairment may be poorly compliant with taking high-dose diuretics or potassium lowering agents. Patients with advanced cardiac disease and low eGFR may have trouble with removing the excess fluid from their bodies, which accumulates as a result of the reduced renal excretion. Once these patients are on HD treatment, frequent dialysis therapy prevents potassium and fluid disbalance[7].

The survival of the ESRD patient depends greatly on their nutritional status and serum albumin levels at the time of HD initiation. Patients that started early on HD (at higher eGFR levels) have a better nutritional status and higher serum albumin levels than those that start later. Protein intake starts to decrease already in early stages of CKD (when eGFR is still above 25 ml/min/1.73m²). Several studies have demonstrated that an early initiation of HD results in decreased hospitalization and mortality rates[8-11]. However, survival may mistakenly appear to be prolonged in patients who initiate HD early if survival is measured from the time of initiation of HD. The patients that start earlier with HD have higher eGFR, however their eGFR still drops while they are on HD. In the period that these patients are on HD and still have higher eGFR than the patients that started with lower eGFR the hospitalization and mortality rate are lower than in the patients that have started HD with lower eGFR. However, it has been proven that when the eGFR of the "early starters" drop to the levels of "late starters" the hospitalization and mortality rates of "early starters" become equal to the rates of "late starters"[12;13]. This means that hospitalization and mortality rates depend on the level of eGFR, not on the time of HD initiation. The statistical error that gave the false impression that initiation of HD a higher level of eGFR lead to reduced hospitalization and mortality rates is known as the "lead time bias".

Advanced Glycation End-Products

The solutes that need to be removed by HD in case of ESRD are so-called uremic toxins. Depending on the molecular size and properties the uremic toxins can be divided

into 3 major classes: small solutes (< 500 D), middle molecules (≥ 500 D), and solutes with known or likely protein binding. The standard HD removes mostly small solutes and middle molecules toxins[14]. The removal of uremic toxins by HD depends on the percentage of the "free" fraction of the uremic toxin in plasma. Substances that are tightly bound to proteins with a low free fraction in the plasma will be removed only to a negligible extent by HD[7].

Advanced Glycation End-products (AGEs) are metabolic end products that belong to the protein-bound uremic toxins[15] meaning that only the "free" AGEs fraction can be removed by standard HD technics.

Figure 3 shows the various pathways that may lead to AGEs formation. Briefly, AGEs accumulation results from hyperglycaemia, hyperlipaemia, oxidative stress, and decreased kidney clearance of AGEs precursors[16]. Classically, AGEs formation has been described as a non-enzymatic reaction between proteins and glucose[17]. Glucose binds with proteins and forms chemically reversible early glycation products that undergo a slow and complex rearrangement to eventually form AGEs. In addition to the formation of glucose-protein intermediates, AGEs are also formed through lipid-derived intermediates, resulting in advanced lipoxidation products[18]. Widely studied AGEs are pentosidine, carboxymethyl-lysine, and carboxyethyl-lysine, which result from oxidation of carbohydrate- and lipid-derived intermediates.
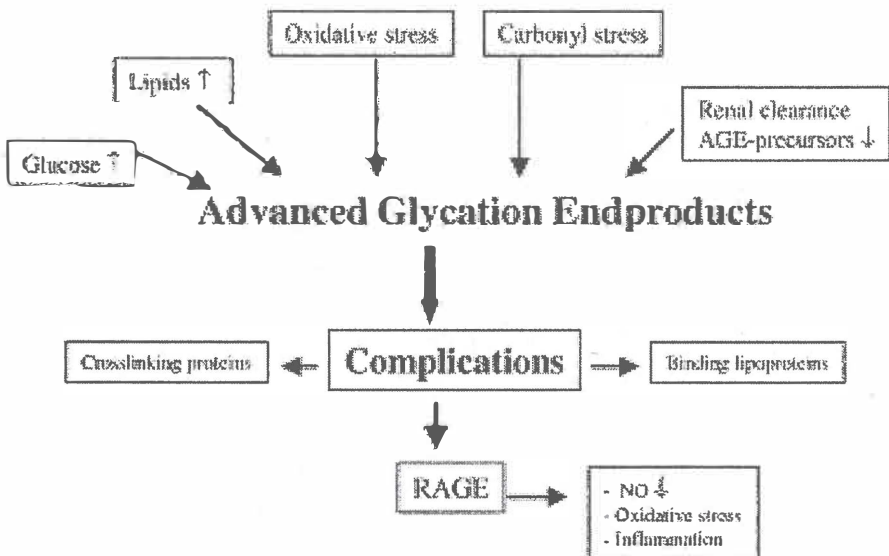
Figure 3. Pathways of Advanced Glycation End-Product formation[18]

After AGEs have been formed, they may be removed by the kidney or they accumulate in tissue where they cross-link with polymers e.g. collagen or interact with specific receptors,

inducing tissue stiffening of blood vessels and skin. As a consequence of poor kidney function the highest AGEs levels are observed in ESRD patients[19]. Moreover, current HD techniques are unfortunately only able to clear a portion of the AGEs from plasma[20].

During a long-term follow-up, skin AGE levels proved to be strong predictors of survival in HD patients[21], independently of other established risk factors like age, existing cardiovascular diseases, serum albumin, and C-reactive protein. Part of the AGEs possesses autofluorescent properties that can be measured in the skin using optical techniques.

The measurement of skin AGEs with the AGE Reader

Skin AGEs can be measured with the AGE Reader. DiagnOptics Technologies B.V., a spin-off company of the University Medical Center Groningen, the Netherlands, has developed this instrument (figure 4). This diagnostic device consists of a box with a built in light source and spectrometer. The measuring unit is connected to a personal computer via a USB connection. A special software program performs a completely automatic measurement of the autofluorescent properties of the skin of the forearm.
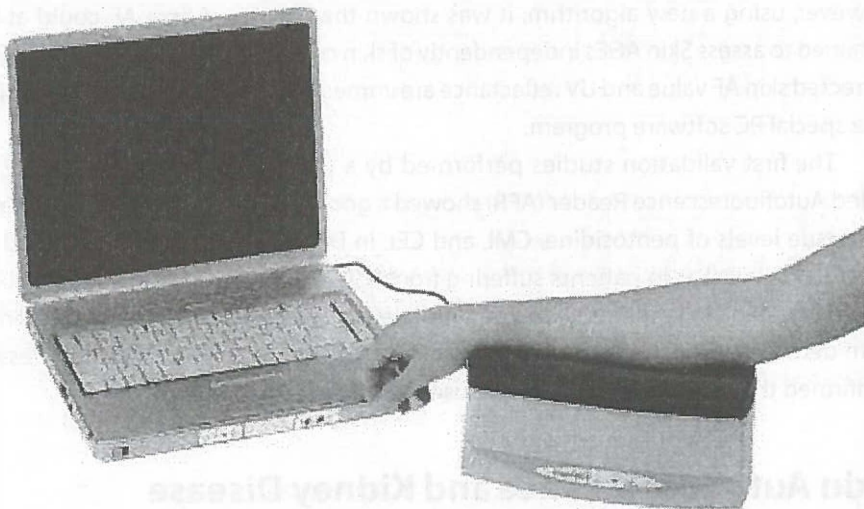
Figure 4. The current version of the AGE Reader as validated device with CE certification since 2006[21]

The working principle of the AGE Reader is as follows: a blacklight tube with a peak wavelength of 370 nm illuminates approximately 4 cm² of the skin of the volar side of the forearm. A non-contact optical fiber detects the emission as well as the reflection of the

excited light at an angle of 45°. Using a spectrometer and computer software, the intensity spectrum is analyzed. Skin AF is calculated from these results as

$$AF = \frac{I_{em}}{I_{exc}} \times 100,$$ (1)

where I_{exc} is the reflected excitation light from a light source in the 300-420 nm range with a peak intensity around 370 nm and I_{em} is the emitted light from the skin, the average light intensity as measured in the 420-600 nm range. Skin AF is expressed in arbitrary units (AU), using the multiplier of 100 to yield manageable values. Besides the Skin AF measurement, a diffuse reflection spectrum in the excitation range is obtained by normalizing the measured spectrum to a spectrum measured on a white reflection standard. To obtain a reflection spectrum in the visible range, a white emitting diode (LED) has been added as illumination source. Information on the diffuse reflection in the visible part of the spectrum has been used by the manufacturer to obtain additional information about the skin color, which is used to improve the accuracy and interpretation of the skin AF measurements. Initially, Skin AF results were applicable in Caucasian subjects only with a UV skin reflection > 10%. However, using a new algorithm, it was shown that results of Skin AF could at least be obtained to assess Skin AGEs independently of skin color for UV reflections above 6%[23]. The corrected skin AF value and UV reflectance are immediately calculated, stored and displayed by a special PC software program.

The first validation studies performed by a prototype of the AGE Reader, the so-called Autofluorescence Reader (AFR) showed a good correlation between the Skin AF and the tissue levels of pentosidine, CML and CEL in DM patients and age-matched healthy controls[24], as well as in patients suffering from ESRD[21]. In a pooled analysis, also including a study by den Hollander et al in younger healthy controls[25], tissue levels of pentosidine from dermal biopsies could explain 76% of the variation in skin AF values[16]. These studies confirmed that the AGE Reader can be used to measure skin AGEs.

Skin Autofluorescence and Kidney Disease

Skin AF was shown to be a strong and independent predictor of overall and cardiovascular mortality in a Dutch population of HD patients[21]. Skin AF increased as eGFR decreased and was related to CVD history in CKD patients[26]. Increased Skin AF was also related to the presence of CVD in Asian (non-Caucasian) HD patients[27]. Diastolic dysfunction in HD patients, assessed using ultrasound Doppler imaging, was related with an increase in Skin AF as well[28]. This is because conditions with a high level of AGEs lead to cardiac stiffness as a result of glycosylation of the heart. The cardiac stiffness leads to reduced cardiac compliance, the property of the heart to expand without an increase in pressure

when blood comes rushing into the ventricles. The reduced compliance results in elevated diastolic pressure in the ventricles what is the main characteristic of the diastolic dysfunction.

Skin Autofluorescence and Diabetes Mellitus

Most of the studies on AGEs have been performed in patients with diabetes. CKD is well known complication of diabetes, which implies that many subjects on HD are patients with diabetes.

In diabetic patients the glucose metabolism is hampered, resulting in increased AGE formation. In a large group of patients with type 2 diabetes (n=973), Lutgers et al. demonstrated that their Skin AF was higher than in healthy control subjects. Skin AF was significantly higher in the type 2 diabetes group with both micro- and macrovascular disorders, as compared with the group without complications and the group with only microvascular complications[29]. Samborski et al. confirmed the results of Lutgers et al, showing that Skin AF in patients with diabetes is significantly higher than in controls. Furthermore they found that there is a significant positive correlation between Skin AF and diabetes duration, and between Skin AF and glycated hemoglobin concentration (HbA1c)[30].

In type 2 diabetic patients it was demonstrated that HbA1c assessments over time can predict the change in Skin AGE levels measured by Skin AF. Also, it was observed that the measurement of Skin AF is an independent predictor of the development of microvascular complications in type 2 diabetes[31]. Monami et al. found that after adjusting for age and HbA1c, micro- or macrovascular complications of diabetes were still associated with higher Skin AF[32].

Multiple regression analysis showed significant correlation of Skin AF with age, sex, diabetes duration, Body Mass Index (BMI), smoking, glycated hemoglobin-HbA1c, plasma creatinine, HDL cholesterol, and albumin-to-creatinine ratio in the type 2 diabetes group[29]. Furthermore, Skin AF correlates with the severity of peripheral and autonomic nerve abnormalities in diabetes, even before being clinically manifest[33]. In a follow-up study, the independent predicative value of Skin AF for macrovascular and microvascular complications was unequivocally demonstrated[34;35]. Moreover, it was shown that Skin AF provides additional information on the risk of subjects with diabetes compared to the existing risk analysis by the United Kingdom Prospective Diabetes Study (UKPDS) risk engine. This resulted in risk-reclassification of a substantial number of patients and identifying patients with particularly high risk for developing cardiovascular events[34].

Skin Autofluorescence and Other conditions

Skin AF was higher in smokers than in non-smokers[36;37]. There is an interaction of smoking and gender, women seem to be more susceptible to the effect of smoking on Skin

AF than men[37]. Furthermore, Skin AF increased as a function of age[21;37;38]. Maury et al. found that Skin AF is higher in the patients with liver cirrhosis as compared to control subjects and that the icteric patients had lower Skin AF values than those without icterus[39].

It was also shown that Skin AF was elevated in stable coronary artery disease and that it was related to the serum level of the soluble receptor for AGEs[40]. Finally, Skin AF was also elevated in patients with ST-elevation myocardial infarction (STEMI), Skin AF was associated with inflammation and glycemic stress, and showed that it can predict future major adverse cardiac events in STEMI patients[41].

Aim of the thesis

This thesis is focused on behavior of AGEs in the skin of HD patients. Some of the questions that we have tried to address are:

- Which method of measuring AGEs has greater clinical value, Skin AF or direct skin biopsies measurements?
- How does the HD treatment affect the level of AGEs in plasma and skin measured by AF?
- Does Hepatitis C infection result in increased AGEs accumulation in HD patients?
- What is the accumulation rate of AGEs in the tissue of HD patients?
- Can the rate of AGEs accumulation be used as a predictor of survival of HD patients?
- Can AGE food intake and BMI influence the rate of AGEs accumulation in HD patients?

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Chapter 2

Advanced Glycation End-Products and Skin Autofluorescence in Chronic Kidney Disease: a Review

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Abstract

Chronic Kidney Disease (CKD), especially in its end stage, is marked by extremely high cardiovascular rates of morbidity and mortality; hemodialysis patients have a 5-fold shorter life expectancy than healthy subjects of the same age. In CKD the metabolic products that accumulate in the body are so-called uremic toxins. These include Advanced Glycation End-products (AGE). AGE levels are markedly increased in CKD patients not only because of impaired excretion but also because of increased production. AGE formation has initially been described as a non-enzymatic reaction between proteins and glucose in the so-called Maillard reaction, but they are also more rapidly formed during oxidative stress and subsequent formation of reactive carbonyl compounds like (methyl)glyoxal. AGE accumulate in tissue where they cross-link with proteins e.g. collagen, inducing tissue stiffening of blood vessels and skin. They may also interact with receptor of AGE (RAGE) and other receptors, which lead to activation of intracellular transduction mechanisms resulting in cytokine release and further tissue damage in CKD. The accumulation of AGE in the skin can be measured non-invasively using autoflorescence. The skin autofluorescence is a strong marker of cardiovascular mortality in CKD. The focus of this review is on the role of tissue and plasma AGE, and of skin autofluorescence as a proxy of tissue AGE accumulation, in the increase in cardiovascular disease in end stage renal disease (ESRD). This review will also present the possibility of reducing the AGE accumulation in ESRD patients using the following five methods: 1. use of low AGE peritoneal dialysis solutions; 2. use of advanced hemodialysis techniques; 3. use of AGE reducing drugs; 4. optimizing the nutrition of hemodialysis patients; and 5. renal transplantation.

Introduction

Chronic Kidney Disease (CKD) encompasses a wide clinical spectrum of conditions, both in degree of renal function loss and in pathogenesis. The degree of renal function loss is currently divided in 5 CKD classes according to the level of the (estimated) glomerular filtration rate (eGFR). Mild CKD (CKD 1-2) is very common in elderly but may also be present in some primary glomerular diseases with other more prominent features like proteinuria. More advanced loss of renal function finally resulting in end-stage renal disease (ESRD) or CKD 5 is less common, with atherosclerosis-associated glomerulosclerosis and diabetic kidney disease as the most prominent causes.

It is widely accepted that the focus of monitoring and treating CKD is on prevention of not only progressive loss of renal function, but also of the marked increase in cardiovascular disease (CVD) and death. The extremely high CVD rate in CKD 4-5 and dialysis patients forms the most impressive illustration: hemodialysis (HD) patients have a 5-fold shorter life expectancy than healthy subjects of the same age. The leading cause of death in patients with ESRD is CVD [1].

In CKD the metabolic products that accumulate in the body are so-called uremic toxins. These include Advanced Glycation End-products (AGE) [2]. AGE levels are not just markedly increased in CKD patients due to increased production, they are also due to impaired excretion [3]. Classically, AGE formation has been described as a non-enzymatic reaction between proteins and glucose in the Maillard reaction [4]. Glucose binds with proteins and forms chemically reversible early glycation products that undergo a slow and complex rearrangement eventually forming AGE. In addition to the formation from glucose-protein intermediates, AGE are also formed through lipid-derived intermediates, resulting in advanced lipoxidation products [5]. Furthermore, rapid formation of AGE via another pathway involving reactive carbonyl compounds like (methyl)glyoxal (so-called dicarbonyl stress) occurs during oxidative stress [6]. The glyoxalase system forms a defence mechanism against this pathway [7]. Finally, and of special interest in CKD, a source of AGE in humans is the intake of exogenous AGE from food and smoke [8]. When proteins with AGE linked to them are degraded to so-called glycation free adducts and glycation adduct residues of proteins, especially the former are subsequently excreted via the kidney [3]. In the case of renal failure, this excretion mechanism fails or is overridden. AGE will further accumulate in tissue where they cross-link with proteins e.g. collagen, inducing tissue stiffening of blood vessels and skin. They may also interact with receptor of AGE (RAGE) and other receptors, via activation of intracellular transduction mechanisms resulting in cytokine release and further tissue damage in CKD [9].

The focus of this review is on the role of AGE, and of skin autofluorescence (SAF) as a proxy of tissue AGE accumulation, in the increase in CVD in more advanced stages of CKD Moreover, it will propose that the role of AGE and SAF is not restricted to the classical examples of AGE-associated CKD. For a long time, it has been well accepted that diabetic

nephropathy is the classical model for demonstration of the pathogenic role of AGE. However, more recently it has become evident that AGE accumulation also has a role in progression of CKD and in CVD in non-diabetic kidney disease. This will be discussed in the following paragraphs.

Plasma Advanced Glycation End-Products in Chronic Renal Failure

Galli et al. showed progressively higher levels of plasma pentosidine, assessed using high performance liquid chromatography comparing matched groups of healthy controls, CKD, and hemodialysis (HD) patients, respectively. Within the HD group, a negative correlation existed between the level of plasma pentosidine and dialysis frequency. They also proved that protein-leaking HD reduces the level of plasma pentosidine. In renal transplantation patients plasma pentosidine were similar to those in healthy controls[10].

Galli's data are in line with those of other groups, showing that AGE indeed accumulate in non-diabetic uremic patients, despite their normal serum glucose levels. In lower CKD classes, a relation between AGE levels, (Nε-carboxymethyl-lysine-CML), and renal function is also evident, both in selected groups and in the community[11]. Among dialysis patients, both diabetics and non-diabetics have high plasma pentosidine and CML levels. Unfortunately, current hemodialysis (HD) techniques are only able to clear a portion of AGE from plasma[12]. Hou et al. propose that AGE and RAGE may contribute to amplification of inflammation in non-diabetic CKD[13]. Uribarri et al. showed that AGE intake contributes to the level of plasma AGE levels in CKD patients[14].

However, one should be aware that the impact and resulting damage of all these factors that accelerate plasma AGE levels increase are strongly dependent on the behaviour of the molecules and tissues to which the AGE link. The degree of AGE accumulation and resulting damage will be more evident in tissues with slow turnover. In fact, in the commonly used plasma/serum compartment for taking AGE samples, AGE link to proteins with a high turnover rate. Several studies support that plasma/serum AGE may be a poor mirror of AGE dependent tissue damage[15-17]. Although the levels of plasma AGE are very high in ESRD patients, SAF as a mirror of dermal tissue AGE accumulation qualifies as a better marker of tissue damage than plasma AGE in these patients. Ueno et al. reported that in ESRD patients, both SAF and serum pentosidine correlated with carotid intima-media thickness, and SAF also inversely correlated with endothelial progenitor cells, while such a relation was absent for serum pentosidine. In multiple regression analysis, SAF, but not serum pentosidine and intima-media thickness, was related to endothelial progenitor cells[17]. Another example of this dissociation between AGE in plasma and long-lived tissues is a study by Hartog et al. in which plasma AGE and diastolic function were not related, while a strong relation existed with SAF. In this study the diastolic dysfunction of HD patients, assessed using ultrasound

Doppler imaging, was related with the increase in SAF[15]. Furthermore in another study, serum CML did not correlate with CVD in a large group of HD and peritoneal dialysis (PD) patients, whereas SAF did[16].

The Measurement of Skin Advanced Glycation End-Products Using Skin Autofluorescence

SAF can be measured with the AGE Reader (DiagnOptics Technologies BV, Groningen, the Netherlands). The AGE Reader is a desk-top device that uses the characteristic fluorescent properties of certain AGE to quantify the level of AGE accumulation in the skin. In short, the AGE Reader illuminates a skin surface of 4 cm² guarded against surrounding light, with an excitation light source with a peak excitation of 370 nm (ultraviolet A). Emission light (fluorescence in the wavelength of 420–600 nm) and reflected excitation light (with a wavelength of 300–420 nm) from the skin is measured with a spectrometer. SAF is calculated as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units (AU). In validation studies using skin biopsies taken from the site of SAF measurements, a strong correlation was found between SAF and the skin contents of the fluorescent AGE ,pentosidine, as well as with the non-fluorescent AGE, Nɛ-(carboxymethyl)-lysine (CML), and Nɛ-(carboxyethyl)lysine (CEL) [18-20]. One of these validation studies was performed in HD patients[19]. Furthermore a combined analysis performed on the three studies showed that 76% of the variance in SAF may be explained by the associated pentosidine levels[21].

Meerwaldt et al. showed an intra-individual Altman error percentage of 5.03% with SAF measurements taken over 1 single day, and an Altman error percentage of 5.87% for seasonal variation[18]. Similar results were reported in stage III CKD patients by McIntyre et al.[22].

Skin Autofluorescence in Chronic Renal Disease

As previously mentioned above, the behaviour and levels of AGE in the human body are not only dependent on factors like glycaemic and oxidative stress as accelerators of formation of AGE, but also on the presence of intact mechanisms for excretion of AGE free adducts and peptides, which are mainly excreted by the kidney. Thus, loss of renal function in progressive CKD strongly affects these excretion mechanisms, and partly explains the increase in plasma and tissue AGE levels in CKD. SAF increased as eGFR decreased and was related to CVD history in CKD patients[23]. In diabetic nephropathy, increased glycaemic stress may contribute to higher plasma and tissue AGE levels, while in CKD, low-grade oxidative stress is commonly present and enhances AGE formation regardless of diabetic condition.

Skin Autofluorescence in End-Stage Renal Disease
With Renal Replacement Treatment

Additional important factors that increase SAF once renal replacement treatment has started are derived from factors associated with the renal replacement treatment itself, such as the dialysis vintage and the length and amount of glucose exposure in peritoneal dialysis (PD) patients. McIntyre et al. reported that there is not a difference in the level of SAF in hemodialysis and peritoneal patients[24]. Previously, we found a connection between SAF and the presence of diabetes in HD patients[19;25]. Furthermore, the level of SAF of diabetic HD patients is higher than that in diabetic patients with similar age that are not on dialysis[26]. This indicates that ESRD is a separate contributor of AGE accumulation in these patients. Further support has been previously published showing the correlation between SAF and HD vintage[19;25].

Skin Autofluorescence is a Strong Predictor of Cardiovascular Mortality in Chronic Kidney Disease

The Diabetes Control and Complications Trial - Epidemiology of Diabetes Interventions and Complications (DCCT-EDIC) research, has revealed that the reduction in the risk of progressive nephropathy resulting from intensive therapy in patients with type 1 diabetes persist for at least several years after the end of treatment, despite increasing hyperglycemia. In addition, intensive therapy during the DCCT also reduced the risk of cardiovascular events by about 50 % in type 1 diabetic patients 11 years after the end of the trial. In another DCCT-EDIC substudy on biochemically assessed AGE levels in skin biopsies, the higher levels of AGE were found to be independent predictors of worse renal and cardiovacular outcome[27]. These clinical studies strongly suggest that so-called "glycemic memory" causes chronic abnormalities in diabetic vessels that are not easily reversed, even by subsequent, relatively good control of blood glucose. Among various biochemical pathways implicated in diabetic vascular complications, the process of formation and accumulation of AGE and their mode of action are most compatible with the theory "glycemic memory"[28].

McIntyre et al. reported in a cohort of 1707 patients with CKD class 3 that a large number of cardiovascular and renal risk factors were associated with SAF, such as eGFR, hemoglobin, age, smoking, total cholesterol, diastolic blood pressure, c-Reactive Protein, waist to hip ratio, albuminemia, pulse wave velocity, diabetes and uremic acid[22]. In other studies, SAF also tends to be associated either to risk factors of cardiovascular mortality or to direct evidence of cardiovascular damage. Carotid artery intimal-medial thickness was correlated positively with SAF[29]. Skin autofluorescence was also inversely and independently associated with circulating endothelial progenitor cells in ESRD patients[17]. It has been reported that endothelial progenitor cells have the ability to repair cardiovascular damage[30]. In CKD children, tissue accumulation of AGE was observed, aggravated as eGFR declined

and related to early cardiovascular changes and some biochemical CVD risk markers[31].

The clinically most relevant data on the impact of the accumulation of AGE on the cardiovascular system in ESRD patients come from studies that investigated the influence of SAF on cardiovascular morbidity and mortality. Our group was the first to shown that SAF was strong and independent predictor of overall and cardiovascular mortality in ESRD patients[19]. Increased SAF was also related to the presence of CVD in Asian (non-Caucasian) HD patients[23]. Also, a single point SAF measurement is a good predictor of mortality in diabetic patients. Diabetic patients have a higher risk for increased AGE accumulation and mortality than healthy persons[26]. Nevertheless, the presence of diabetes in HD patients did not confer an increased hazard in the multivariate Cox Regression analyses, which was supported by previous studies[19;32]. The explanation for this phenomenon can be that the dialysis procedure interferes much more with the morbidity of the patients and the effect of diabetes is only marginal.

Jiang et al. recently reported on SAF levels a large cohort of 2388 maintenance dialysis patients (613 PD and 1775 HD)[16]. SAF was measured with the AGE Reader. In PD, SAF was strongly correlated with the duration of PD and glucose exposure dose, and independently associated with CVD. In multivariate analysis glucose exposure dose and SAF were the strongest risk factors for CVD in PD, after adjustment for age, gender, and other classic- or uremic-related risk factors. According to Receiver Operator Characteristic (ROC) curve for presence of CVD, the best cutoff point of SAF was determined to be 2.75 AU. Remarkably, this level is the same as used by Lutgers et al. in models for CVD risk reclassification for elderly type 2 diabetes patients[33].

Opportunities for reversibility of the Advanced Glycation End-Products Accumulation

Use of low Advanced Glycation End-Products Peritional dialysis Solutions

During manufacturing, heat is commonly used for sterilization of the PD solutions causing most standard glucose based PD solutions to contain AGE[34]. The duration of PD and the glucose exposure dose are independently associated with the level of SAF in PD patients. Moreover, as reported above, the glucose exposure dose and SAF are the strongest risk factors for CVD in PD[16]. The substitution of glucose with other substances such as icodextrin, a starch-derived branched water-soluble glucose polymer has led even to higher levels of SAF in the PD patients[35].

The problem of increased AGE accumulation as a consequence of the PD treatment might be resolved by use of PD solutions with neutral pH and low AGE content. The use of PD solutions with neutral pH and low AGE content results in lower AGE accumulation, less peritoneal membrane fibrosis and vascular sclerosis[36]. Such PD solutions are available

for more than 10 years on the market; however, the first PD solution of this type has only been just recently approved by the FDA in the USA[34]. Evidence exist that use of PD solutions with neutral pH and low AGE content can result in significant improvement in patient and technique survival without any measurable change in peritonitis incidence[37].

Use of Advanced Hemodialysis techniques

The HD vintage in several studies has been proven to be a contributor to higher levels of SAF, suggesting that ESRD and the HD treatment itself can contribute to AGE formation [19;25]. The use of different HD techniques to ameliorate the AGE accumulation in HD patients has been investigated for some time. A comparison of the removal of free plasma AGE and AGE peptides by low, high, and super flux HD showed that all modalities can effectively remove free plasma AGE during a single HD session. However, plasma protein-bound AGE did not decrease during a dialysis sessions, neither with high flux nor with low flux HD membrane. Super flux has been suggested to be a modality capable of reducing AGE peptides in the long term[38-40]. Another study showed that long-term HD with protein-leaking membrane also reduces predialysis protein-bound and free plasma AGE levels[41]. Also, single dialysis session with on-line hemodiafiltration (HDF) results in lower plasma AGE than with those treated with conventional low and high flux HD. The long term use of HDF provides lower predialysis plasma AGE levels compared with those treated with low and high flux HD[42].

Other factors can reduce the level of plasma AGE in HD patients. The material from which the dialyser is made has an influence on the level of plasma AGE. The use of polysulfone membranes appear to result in lower levels of plasma AGE than using non-polysulfone membranes, such as modified or unmodified cellulose membranes[43]. Also, the use of Vitamin E-coated HD membrane resulted in reduction of plasma AGE levels[44]. The use of ultrapure HD fluid appears to results in lower plasma levers in HD patients. Several studies showed that ultrapure dialysate decreases plasma levels of AGE in HD patients[45;46]. The reduction of plasma AGE by use of ultrapure dialysate is achieved regardless of dialyzer membrane type[47]. The mechanisms of the reduction of plasma AGE by use of ultrapure dialysate is still unknown. Furthermore, one study demonstrated that a daily HD (2h, 6 times/week) regimen can effectively lower the levels of plasma AGE observed in a standard HD (4h, 3 times/week)[48].

Therefore, we can conclude that reduction of the AGE accumulation can be achieved by using advanced HD techniques which use membranes that have large pores such as super flux, HDF or protein leaking HD. The reason is that these techniques are able to filter protein-bound AGE. Also, the use of more biocompatible membranes and ultra-pure HD fluid can reduce the AGE accumulation, most likely by reducing the level of inflammation and immune response. The use of more frequent HD regime such as daily or home dialysis can achieve better removal of plasma AGE and thus lower AGE accumulation. It would be

important to obtain clinical data on the effects of such alternative HD techniques on CVD events and mortality. So far, such data are still lacking.

Use of Advanced Glycation End-Products Reducing Drugs

The use of many herbal medicines and experimental drugs has shown a reduction of AGE accumulation[49-56]. Also, many conventional drugs are useful in the reduction of AGE. SAF levels can be reduced by using angiotensin receptor blockers and statins in PD patients[35]. A calcium channel blocker, azelidipine, has shown to be able to reduce the level of plasma AGE in hypertensive CKD patients[57]. In a single-center, randomized, 2-month, open-label, intention-to-treat, crossover study, sevelamer carbonate, a calcium based phosphate binding drug, was used. Sevelamer blocks the absorption of ingested cytotoxic AGE in the gut. Sevelamer reduced the level of plasma AGE, lipids, and inflammation in diabetic CKD patients in absolute and relative terms when compared with the standard treatment of calcium carbonate[58].

It appears that there are many promising drug treatments and what is even better that already existing approved drug, like Sevelamer can be used in the reduction of the AGE accumulation in patients with CKD.

Optimizing the Nutrition of Hemodialysis Patients

Some controversy remains on the relationship between AGE intake and plasma AGE in humans[59-62]. Uribarri et al.[14] first showed that AGE intake correlates with circulating AGE levels in renal failure patients. However, a single point measurement of SAF did not correlate with the AGE intake of elderly healthy subjects[63]. The relationship between Body Mass Index (BMI) and SAF was also studied. It was found that a single measurement of SAF had a positive correlation with BMI in healthy subjects[64], whereas BMI and SAF did not correlate in HD patients[19].

There is still controversy if a reduction in AGE accumulation can be achieved by reduction of AGE food intake in HD patients. Also, it appears that the nutrition state measured by BMI has some role in the accumulation of AGE in HD patients.

Renal Transplantation

Renal transplantation in theory should be the ultimate treatment of CKD and therefore result in total reversibility of AGE accumulation. Indeed, the levels of protein-bound pentosidine in plasma in renal transplant patients are at the level of healthy subjects[10]. However, our findings showed that SAF of renal transplant patients is still increased when compared to patients with the same age. Also, the SAF was associated with several risk factors for CVD and chronic renal transplant dysfunction in patients with renal transplant[65]. This indicates that a complete reversibility may not easily be obtained because AGE

accumulation in skin and other tissues with slow turnover is not easily reversible. Moreover, on-going oxidative stress due to immunosuppressive treatment, episodes of rejection, and infections may be on-going stimuli for continued AGE accumulation. A latter study showed that SAF remains a very strong determinant of (still markedly increased) mortality and graft function loss[66]. Thus, renal transplantation is remarkably an incomplete solution, and a dire need for slow tissue AGE reversibility remains.

Clinical Perspectives

SAF is a proven marker of CVD mortality in CKD. Nevertheless, clinical nephrologists often state that cardiovascular risk in CKD patients is so high that new risk markers will not change their already intensive treatment policy, and thus SAF or AGE markers have little to add. This may indeed be true for ESRD or HD/PD patients, but it is not valid for the quantitatively important groups of patients with CKD. Availability of tools to select persons in the very prevalent lower CKD classes but at the highest CVD risk is, in our opinion, of major importance in order to focus the conventional CVD preventive treatment. Secondly, newer approaches, such as illustrated by the sevelamer, may still make a difference even in CKD stage IV-V and HD/PD patients and renal transplant patients. Thirdly, the patients in CKD stage IV-V with the highest SAF levels may be those that qualify for priority renal transplant. And finally, the fact that SAF is a very simple tool to provide more insight into the role of AGE (dependent mechanisms) in development of CVD also counts.

Future Prospects

More frequent and repeated measurement of SAF in CKD patients can give insight on many factors that influence the AGE accumulation in these patients. In earlier stages of CKD, more focus is needed on therapeutic options aiming at reduction of AGE accumulation. An obvious possible line of further research can be the influence of drugs such as sevelamer on the accumulation of AGE measured by repeated SAF measurement. Also, the influence of different diets like low AGE diet or optimal nutrition level on the rate of AGE accumulation in early stages of CKD should be investigated. In ESRD, the influence of different renal substitution methods such as super flux HD, HDF, protein leaking HD, daily and home HD or use of ultrapure fluid on the rate of increase of SAF warrant further investigation.

Conclusions

AGE, especially those that are tissue-bound, are not just an important marker of patients' outcome in CKD but also a major contributor to the underlying condition. Therefore, they should be monitored regularly and carefully. Today, there are many methods of tissue

AGE reduction, like use of modern PD and HD techniques, AGE reducing drugs, dietary interventions and renal transplantation

Conflict of interest Statement

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Chapter 3

Rate of accumulation of advanced glycation end-products: repeated skin biopsies and skin autofluorescence measurements in diabetic hemodialysis patients

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Abstract

Objectives: To measure the rate of accumulation of different Advanced Glycation End-products (AGEs) in the skin of diabetic hemodialysis (HD) patients over a period of one year and to find the factors that influence this process.

Methods: Twenty-two diabetic HD patients were enrolled in this study. Skin biopsies were taken from 14 patients twice with an interval of one year. The amount of pentosidine, carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL) in the skin biopsies was measured biochemically. Skin autofluorescence (AF) was measured non-invasively to determine the AGEs accumulation in the skin. Dietary records from the HD patients were obtained to assess the calorie, protein and AGEs intake. Furthermore, Body Mass Index (BMI), as a measure of nutritional state, was obtained.

Results: The pentosidine, CML and CEL content of the biopsies correlated with Skin AF, but all four variables were not significantly different between the two time points. We found that the independent predictors of the one year increase of pentosidine were: one year increase of Skin AF and the Skin AF value at the first time point. The independent predictors of the one year increase of CML were: one year increase of Skin AF, the Skin AF value at the second time point and the 4th quintile of BMI. The independent predictors of the one year increase of CEL were the one year increase of Skin AF and the 4th quintile of BMI. Neither the amount of AGEs measured in the skin biopsies nor their one year increase correlated with the intake of AGEs, proteins and calories.

Conclusions: Biochemically determined skin AGEs of the diabetic HD patients did not show a significant increase in one year time. The rate of Skin AGEs accumulation is independently associated with Skin AF rate of increase and nutritional state.

Introduction

Advanced glycation end-products (AGEs) are formed during glycemic and oxidative stress and the accumulation of these products is increased in diabetes, renal failure and chronic inflammatory conditions[1]. AGEs are associated with the development of long-term complications of ESRD[2]. Skin AGEs levels are strong and independent predictors of the progression of kidney impairment in patients with diabetes[3;4].

There are many different AGEs. Some of the most studied AGEs are Nε-carboxymethyl-lysine (CML), Nε-carboxyethyl-lysine (CEL), pentosidine, 3-deoxyglucosone-(DG)-imidazolone and pyrraline. Some of the AGEs are fluorescent (pentosidine) while others are not (CEL, CML).

Skin Autofluorescence (AF) is a non-invasive method to measure the level of AGEs in tissue[5]. The level of Skin AF at a given moment is an independent predictor of the overall and cardiovascular disease (CVD) mortality in HD patients[6]. An association between Skin AF accumulation and vintage of the HD treatment has been shown[6-9], however to our knowledge no prospective study to directly measure the change in tissue AGEs over time in HD patients has ever been done. We believe that the changes in the tissue AGEs over time should be highest in HD patients with Diabetes.

The aim of this study is to measure the rate of accumulation of different AGEs in the skin of diabetic hemodialysis (HD) patients, directly from biopsies and by means of measurements of Skin AF over a period of one year, and to find the factors that influence their rate of accumulation.

Methods

Twenty-two diabetic HD patients from the Department of Nephrology in Skopje, Macedonia were enrolled in a European collaborative clinical prospective study on Skin AGE accumulation. Skin biopsies of all 22 diabetic HD patients were taken at the start of the study, however of only 14 patient a second sample was taken after one year. Furthermore, their Skin AF was measured twice also at one year interval. Routine blood examinations were done, as well as plasma markers of oxidative stress, inflammation, endothelial activation and cellular injury. The study protocol was approved by an Ethics Committee, and written informed consent was obtained from each patient. The following exclusion criteria were used: less than 3 months on HD; acute illness or hospitalization three weeks prior to the start of the study; neoplasm; previous kidney transplantation.

Patient characteristics

The presence of CVD, hypertension and diabetes were defined using the following criteria: A patient was considered to have CVD when signs of coronary heart disease,

peripheral vascular disease, or cerebrovascular disease were demonstrated, (International Classification of Diseases, Tenth Revision, Clinical Modification codes I20, I21, I63, I70, and I73)[10].

Hypertension was defined as a systolic blood pressure of >140 mmHg or a diastolic pressure of >90 mmHg measured on at least three different occasions[11]. Also patients under antihypertensive medication were regarded as hypertensive patients.

Diabetes was defined by conventional American Diabetes Association criteria[12].

HD vintage was defined as the duration of the period between the initiation of long-term HD treatment and the start of the study.

Dialysis treatment

All patients underwent HD treatment three times per week with a median session duration of 4 hours. The duration of the HD session was individually adjusted, according to Kidney Disease Outcomes Quality Initiative guidelines, to maintain an equilibrated Kt/V >1.2. The patients were dialyzed with a non-glucose containing bicarbonate dialysis solutions (Aminal 100B + Aminal 100CK, Alkaloid, Skopje, Macedonia) using one of the available low-flux polysulfone (F10 HPS-surface area 2.2 m², Fresenius, Bad Homburg, Germany) or polyamide (Poliflux 6L-surface area 1.4 m², Gambro, Lund, Sweden) dialyzer membranes. The blood flow of the HD device was aimed to be at 300 ml/min and the dialysate flow was 500 ml/min. Patients received standard medical care as appropriate for HD patients.

Skin Biopsies

From the 169 prevalent HD patients in the Department of Nephrology in Skopje, Macedonia 41 (24 %) were with diabetes. We found 22 diabetic HD volunteers that were willing to participate in our study, provided that the biopsies were taken from the shoulder region and not from the forearm in order not to influence the vascular anastomosis and prevent the possible infection in that region. At two time points one year apart, full-thickness excision skin biopsies (≈5X5 mm) were taken from the scapular region under 2% lidocaine local anesthesia. The skin samples were frozen in liquid nitrogen and subsequently stored at −80 °C. Approximately, one year later second skin biopsies were taken in the same manner from the opposite scapular region.

Pentosidine levels in the tissue biopsies were measured by high performance liquid chromatography (HPLC)[13]. Tissue proteins were hydrolysed in 6 N HCL. Separation was done with a run-to-run time of 30 min, on a C18 Allspehere ODS-II column with a citric acid acetonitrile buffer. Detection of pentosidine was based on its own fluorescence characteristics using fluorimetric detection (Ex = 325 nm, Em = 385 nm). This detection enables sensitive and specific determination of pentosidine with a detection limit of 2.2 nmol/L or 0.02 pmol/mg. CML and CEL were determined by stable-isotope-dilution liquid chromatography tandem mass spectrometry (SLC/MS)[14]. CML and CEL were liberated from

plasma proteins by acid hydrolysis after addition of deuterated CML and CEL as internal standards. Chromatographic separation was performed by gradient-elution reversed-phase chromatography with a mobile phase containing 5 mmol/L nonafluoropentanoic acid as ion-pairing agent. Mass transitions of 205.1→84.1 and 219.1→84.1 for CML and CEL, respectively, and 209.1→88.1 and 223.1→88.1 for their respective internal standards were monitored in positive-ion mode. The measurements were normalized to the amount of lysine as a marker of the protein content of the skin sample. These measurements were done at University Medical Center Maastricht.

Skin Autofluorescence

Skin AF of the forearm was measured using an AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands). The AGE-Reader illuminates a skin surface of approximately 4 cm² with excitation light mainly between 350 and 420 nm (peak excitation approximately 370 nm). The measured Skin AF is the average reflected light intensity between 420 - 600 nm, divided by the amount of reflected light between 300 - 420 nm, multiplied by 100, Skin AF = reflected light (420 - 600 nm) / reflected light (300 – 420 nm) * 100. In order to correct Skin AF as a function of skin colour, reflection measurements with a white LED were obtained with the AGE Reader as well. Skin AF is measured in arbitrary unites (AU). The measurements were performed in triplicate at room temperature in a room with windows covered by curtains to avoid disturbances by sun illumination (semi-dark). The median value of the triple measurement was used for further analysis. We used AGE Reader software version 2.3 that included correction for dark skin color[15]. Skin AF measurements we done twice with a period of one year between them.

Biochemical blood analyses

The routine monthly analysis that was performed less than 15 days from the day of Skin AF measurements was used in our study. The following routine laboratory parameters were used: urea, creatinine, sodium (Na+), potassium (K+), calcium (Ca++), phosphates, hemoglobin concentration, platelets count, alkaline phosphates (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutathione-S-transferase (γ-GST), lactate dehydrogenase (LDH), plasma total proteins, plasma albumin, direct and total bilirubin, uric acid and hepatitis-B-virus surface antigen (HBsAg). All these values were applied in the statistical analysis of the results.

Additional blood analysis of the concentration of Superoxide Dismutase (SOD), Myeloperoxydase (MPO), high sensitive C-Reactive Protein (hs-CRP), von Willebrand Factor (vWF), Inter-Cellular Adhesion Molecule 1 (ICAM-1) and Heart-type Fatty Acid Binding Protein (H-FABP) in blood plasma were performed. The methods used in the additional analysis have been previously described in detail[16].

The blood samples for the additional blood analysis were taken on the day of the Skin AF measurements. All blood samples were taken from the arterial line of the vascular access at the end of the HD session. In a previous study we have confirmed that the concentrations of the measured plasma markers are not affected by the HD session therefore they were allowed to be measured at the end of the HD session[9].

Nutritional Records

Two dietary records, one in autumn and one in spring, from the diabetic HD patients were obtained to assess the calorie, protein and AGE intake per year and to investigate the existence of possible seasonal variation. The dietary records from these patients were obtained for 7 consecutive days. The method of calculation the amount of AGEs in the food was recently submitted for publication. The amount of calories and proteins was calculated using tables from The United States Department of Agriculture Nutrition's Database[17].

Body Mass Index (BMI), as a measure of nutritional state, was calculated as a ratio of dry weight in kilograms (end-dialysis weight) to height squared (in square meters)[18].

Statistical Analyses

Comparisons between the variables were performed with paired or independent Student t-test depending of the relation between the variables. If the data were skewed a $\log_{10}$ transformation was used. The correlations were analyzed with Spearman rank method. Multiple linear regression analysis was used to find the independent factors of AGE accumulation. In the linear multiple regression models we used only the variables that had $p < 0.10$ in the univariate analysis. Two-tailed $p < 0.05$ was considered significant. Data are shown as mean ± standard deviation.

Results

The mean age of the 22 diabetic HD patients at the start of this study was 60 years (range 43 - 82). The mean HD vintage was 5.1 years (range 1.1 - 20.8). The weekly average dialysis duration was 12.2 hours (range 9 - 15). Seven diabetic HD patients (32 %) had CVD. The patient characteristics are listed in Table 1. From the additional blood analysis SOD, MPO, ICAM-1, hs-CRP and H-FABP were higher than the reference values at the start of the study; the values are listed in Table 2.

Table 1. Characteristics of all 22 diabetic HD patients at the beginning of the study

Variable	
Gender (Male)	15 (68%)
Age (years)	60.3±9.2
Smokers	2 (9%)
CVD history	7 (32%)
HbSAg	2 (9%)
Hypertension	4 (18%)
HD vintage (years)	5.10±5.04
Average weekly duration of the HD treatment (hours)	12.2±0.46

CVD- Cardiovascular Disease; HbSAg- Hepatitis B Surface Antigen; HD- Hemodialysis

Table 2. Additional Blood analysis at the beginning of the study

Variable	Reference Value	This study
SOD (ng/ml)	48±2[35]	480 ± 260
MPO (ng/ml)	43.80± 23.30[36]	600 ± 306
hs-CRP (µg/ml)	<5	12 ± 26
ICAM-1(ng/ml)	0.111± 0.017[37]	2.3 ± 0.8
vWF (%pool)	50–166%	140 ± 52
H-FABP (ng/ml)	4.4±3.9[38]	37±34

SOD- Superoxide Dismutase; MPO- Myeloperoxydas; ICAM-1- Inter-Cellular Adhesion Molecule-1; hs-CRP- High
Sensitive C-Reactive Protein; H-FABP- Heart-type Fatty Acid Binding Protein; vWF- von Willebrand Factor

AGEs Content and Skin Autofluorescence

Using paired t-test we compared the pentosidine, CML and CEL content of the diabetic HD patients at 12 month interval. The pentosidine, CML and CEL were not significantly different between the time points. Also the Skin AF did not significantly change in the period of 12 months between the time points as shown in Table 3. The levels of pentosidine, CML and CEL at the first time point correlated with each other; the strongest correlation was between pentosidine and CML ($R = 0.939$ $p < 0.0001$), the less strong correlation was between CML and CEL ($R = 0.852$ $p < 0.001$) and the weakest was between pentosidine and CEL ($R = 0.688$ $p < 0.001$). At the second time point also the strongest correlation was between pentosidine and CML ($R = 0.883$ $p < 0.001$) the second also was between CML and CEL ($R = 0.641$ $p = 0.02$) whereas the correlation between pentosidine and CEL was not significant.

In the univariate linear regression we found that pentosidine, CML and CEL correlate with the Skin AF at the start of the study (R = 0.547 p = 0.02, R = 0.534 p = 0.02 and R = 0.443 p = 0.04, respectively). Multivariate linear regression analysis showed that independent markers of the level of pentosidine at the start of the study were: Skin AF and LDH, (R = 0.781; p = 0.01). Independent predictors of CML at the start of the study were: Skin AF and MPO (R = 0.694; p = 0.01) whereas the independent predictor of CEL at the start of study was Skin AF and 4th Quintile of BMI (R =0.639; p =0.02).

Table 3. Advanced Glycation End-Products at 2 time points 12 months apart

	Pentosidine (nmol/mmol Lysine)	CML (nmol/mmol Lysine)	CEL (nmol/mmol Lysine)	Skin AF (AU)
Time Point 1	43.7±29.1	942±362	337±74.4	3.20± 0.93
Time Point 2	37.4±12.1	874±175	327±66.5	3.50±0.76
p-value T-test (paired)	0.449	0.526	0.659	0.093

CML- Carboxyymethyl-lysine; CEL- Carboxyethyl-lysine; AF- Autoflorescence

Furthermore, linear multiple regression analysis of the factors that influence the one year increase of pentosidine, CML and CEL was performed. We found that independent predictors of the one year increase of pentosidine were: one year increase of Skin AF and Skin AF at the first time point (Table 4). The independent predictors of the one year increase of CML were: one year increase of Skin AF, Skin AF at the second time point and 4th quintile of BMI (Table 5). The independent predictors of the one year increase of CEL were the one year increase Skin AF and 4th Quintile of BMI (Table 6).

AGEs content and nutritional analysis

The daily AGE intake 8.7 ± 3.6 MU/day of the autumn record did not significantly differ from the value in the spring record, 9.6 ± 3.4 MU/day. The protein intake was 1.5 ± 0.9 and 1.4 ± 0.7 g/kg of body weight per day respectively, which was slightly above the upper limit of the KDOQI guidelines which recommend a protein intake of 1.2 to 1.3 g/kg body weight per day [19]. Furthermore, the calorie intake of 26.4 ± 7.3 in the first record and 27.3 ± 5.4 Kcal/kg body weight per day in second record were slightly below KDOQI guidelines of 30-35 Kcal/kg bodyweight per day for HD patients [19].

The pentosidine, CML and CEL content of the skin biopsies did neither correlate with the AGE, protein and calorie intake at the first nor at the second time point. Furthermore,

the one year increase of pentosidine, CML and CEL of the skin biopsies did not correlate with the AGE, protein and calorie intake

Table 4. One year increase of skin Pentosidine (nmol/mmol Lysine) and demographic, biochemical and other parameters of diabetic hemodialysis patients

Variable	Univariate Analysis		Multivariate Analysis	
	R value	p-value	R value	p-Value
One year increase of Skin AF	0.728	0.017	0.687	0.003
CVD	-0.627	0.039		
Skin AF T1	0.796	0.006	0.598	0.007
Skin AF T2	-0.648	0.043		
BMI 4th Quintile	-0.646	0.032		
Glucose	-0.673	0.023		
LDH	0.682	0.021		
vWF	0.655	0.040		
hs-CRP	-0.682	0.021		

AF- Autofluorescence; CVD- Cardiovascular Disease; BMI- Body Mass Index; LDH- Lactate Dehydogenase; vWF- von Willenbrand Factor; hs-CRP- High Sensitive C-Reactive Protein;

Table 5. One year increase of skin Nε-carboxymethyl-lysine (nmol/mmol Lysine) and demographic, biochemical and other parameters of diabetic hemodialysis patients

Variable	Univariate Analysis		Multivariate Analysis	
	R value	p-value	R value	p-Value
One year increase of Skin AF	0.721	0.019	0.641	0.001
CVD prevalence	-0.689	0.019		
Skin AF T2	0.625	0.053	0.444	0.007
BMI 4th Quintile	-0.595	0.053	-0.437	0.014
Trombocites	-0.848	0.033		
LDH	0.711	0.014		
hs-CRP	-0.710	0.014		
SOD	0.553	0.078		

AF- Autofluorescence; CVD- Cardiovascular Disease; BMI- Body Mass Index; LDH- Lactate Dehydogenase; hs-CRP- High Sensitive C-Reactive Protein; SOD- Superoxide Dismutase;

Table 6. One year increase of skin Nε-carboxyethyl-lysine (nmol/mmol Lysine) and demo-graphic, biochemical and other parameters in diabetic hemodialysis patients

Variable	Univariate Analysis		Multivariate Analysis	
	R value	p-value	R value	p-Value
One year increase of Skin AF	0.799	0.006	0.893	0.007
Skin AF T1	0.762	0.010		
BMI 4th Quintile	-0.643	0.033	-0.677	0.030
LDH	0.745	0.009		
ICAM-1	0.619	0.042		
vWF	0.642	0.045		

AF- Autofluorescence; BMI- Body Mass Index; ICAM-1- Inter-Cellular Adhesion Molecule-1; vWF- von Willebrand Factor;

Nutritional Analysis

The daily AGE intake 8.7 ± 3.6 MU/day of the autumn record did not significantly differ from the value in the spring record, 9.6 ± 3.4 MU/day. The protein intake was 1.5 ± 0.9 and 1.4 ± 0.7 g/kg of body weight per day respectively, which was slightly above the upper limit of the KDOQI guidelines which recommend a protein intake of 1.2 to 1.3 g/kg body weight per day[22]. Furthermore, the calorie intake of 26.4 ± 7.3 in the first record and 27.3 ± 5.4 Kcal/kg body weight per day in second record were slightly below KDOQI guidelines of 30-35 Kcal/kg bodyweight per day for HD patients[22]. The AGE intake as well as the protein and calorie intake was not significantly different between the autumn and spring records. Therefore, we can conclude that the food intake was rather stable during the year.

Patients` Mortality

In the time period of 36 months of follow-up 9 diabetic HD patients died (41 %): 6 from CVD, 3 from non-CVD (infections and liver failure). Univariate Cox regression analysis was applied to overall mortality, using all clinical, demographical, biochemical and nutritional parameters, and the Skin AF and skin biopsies AGEs measurements. The variables that had a $p \leq 0.10$ in the univariate Cox regression analysis were included in the multivariate Cox regression. In the multiple Cox regression analysis of mortality the Skin AF at the start of the study, one year increase of Skin AF and hs-CRP appeared to be the independent predictors (Table 7).

Discussion

In the present study we measured skin AGEs directly using HPLC and SLC/MS, as well as with Skin AF at one year interval in diabetic HD patients. To our surprise we did not note a significant change in level of skin AGEs as determined from the biopsies and Skin AF between the measurements at one year interval in our group of diabetic HD patients. This can be due to the small size of the group.

In validation studies using skin biopsies taken from the site of Skin AF measurements, a strong correlation was found between Skin AF and the skin contents of pentosidine, CEL and CML in healthy subjects, glycogen storage disease and in HD patients[5;6;20]. In our study the skin levels of pentosidine, CEL and CML measured at the scapular region correlated only slightly weaker with Skin AF measurement at the forearm than in the studies mentioned above. The only weaker correlation between Skin AF and direct skin AGEs measurements at different locations than at the same location suggests that the skin AGEs accumulation is relatively uniform on the body.

Furthermore, we found that the one year increase of CML and CEL is influenced by the BMI, the 4th quintile of BMI (22.1 - 25.9 kg/m²) resulted in lower one year increase of the Skin AGEs. This is in agreement with the results of a previous study in which we found a relationship between BMI and the one year increase of Skin AF, which can be represented by a U-shaped curve with the lowest point at a BMI of 24.3 kg/m². These two studies indicate that there is an optimal level of BMI around the WHO upper limit that results in lowest rate of AGEs accumulation.

The mean daily AGE intake of 9.6 MU/day in the present study was 40% lower when compared to the AGE intake of HD patients in a study in the United States who had an average AGE intake of 16 MU/day[21]. Vlassara et al[22] and Negrean et al[23] described prospective studies using a low AGE diet of 3 - 4 MU/day and a high AGE diet of 15 - 16 MU/day. In this regard the daily AGE intake of our study group was intermediate to those studies.

Some controversy remains on the relationship between AGE intake and plasma AGE in humans[22;24-26]. Uribarri et al[21] first showed that AGE intake correlates with circulating AGE levels in renal failure patients. Others found that a single point measurement of Skin AGEs, using Skin AF, was not correlated with the AGE intake of elderly healthy subjects[27]. In a recent study in our Skopje dialysis center we found that the one year increase of Skin AF had a borderline negative correlation with AGE and calorie intake. In the present study the AGEs in the skin measured by HPLC and (SLC/MS) did not correlate with the AGE, protein and calorie intake in diabetic HD patients.

Limitations

The number of diabetic HD patients on which skin AGEs measurements were performed was only 22; therefore the power of the study was limited. A low number of

recruited diabetic HD patients could be expected due to the invasive nature of the skin AGEs measurements.

The assessments of the dietary intake were performed at two time points with 6 months interval and were not performed at the same time as the Skin AF and AGEs measurement. We assumed that no seasonal changes in the dietary intake of AGE, calories and proteins were present.

Conclusions

Skin AGEs of the diabetic HD patients measured by Skin AF and direct AGEs methods did not show a significant increase in one year time. The rate of Skin AGEs accumulation is independently associated with Skin AF, the rate of increase of Skin AF and nutritional state (BMI).

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Chapter 4

Skin autofluorescence, a Measure of Cumulative Metabolic stress and Advanced Glycation End Products, is not changed by the decrease in plasma fluorescence during haemodialysis.

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Abstract

Background and objectives: Tissue advanced glycation end products (AGE) are products of cumulative metabolic and oxidative stress and inflammatory reactions. AGE contribute to the cardiovascular complications of haemodialysis (HD) patients. Skin autofluorescence (AF) is related to the accumulation of AGE, and is one of the strongest prognostic markers of mortality in these patients. The aim was to investigate whether changes in Skin AF could be observed after a single HD treatment.

Design, setting, participants & measurements: A clinical prospective interventional study was performed on 35 patients on maintenance HD therapy (9 women and 26 men, median age 68 years, range 33-83) at the University Hospital in Umea. Median dialysis time was 4 hours (range 3-5.5). Skin and Plasma AF measurements were performed before and after HD.

Skin AF was measured non-invasively with an AGE Reader before and after HD treatment using dialysate that contained 5 mmol/l of glucose. Plasma AF was measured at 460 nm after excitation at 370 nm.

Results: The HD patients had on average a 65% higher Skin AF value than age-matched healthy persons ($p < 0.001$). Plasma AF was reduced by 14% ($p < 0.001$), whereas Skin AF was not changed after a single HD treatment.

Conclusions: This study showed that although Plasma AF decreased significantly after a single HD session, this did not lead to instant differences in the cardiovascular risk marker Skin AF. Nevertheless, adequate HD remains important also from the point of view of clearance of solutes that otherwise contribute to the progression of cardiovascular morbidity.

Introduction

Advanced glycation end products (AGE) are products of cumulative metabolic and oxidative stress and inflammatory reactions. AGE accumulation in tissue is thought to contribute to the chronic complications of diabetes and end stage renal disease (ESRD), such as dialysis amyloidosis and cardiovascular diseases[1-8].

AGE accumulates in the body as a result of a combination of hyperglycaemia, oxidative and carbonyl stress, and decreased renal clearance of AGE precursors. The carbonyl stress theory formulates that increased oxidation of carbohydrates and lipids and/or inadequate detoxification of carbonyl compounds may contribute to the formation of AGEs. AGEs act directly to induce cross-linking of long-lived proteins such as collagen to promote vascular stiffness and thus alter vascular structure and function. Interaction with receptors, such as the receptor for AGE (RAGE), induces intracellular signalling that leads to enhanced oxidative stress and elaboration of key pro-inflammatory and prosclerotic cytokines[8].

Well-functioning kidneys are important organs that contribute in the elimination of AGE, whereas AGE are significantly increased in uraemia even in the absence of hyperglycaemia. The increased accumulation of AGE that is found in HD patients is related to the lack of both breakdown and removal of AGE by the impaired kidneys[9]. Therefore, a progressive chronic kidney disease will increase the risk for accumulation of such substances. The consequence of such retention will be a progressive damage of e.g. vascular cells such as endothelial cells, and subsequent a progress of atherosclerosis and cardiovascular disease[8; 10-12].

Tissue autofluorescence has shown to be related to the accumulation of AGE in the skin[13]. Until recently, skin AGE could only be determined from biopsies. Nowadays, a new non-invasive optical tool, the AGE Reader (DiagnOptics, Groningen, The Netherlands) is available to measure skin autofluorescence (Skin AF), which makes it possible to recognize the state of increased skin AGE non-invasively[14; 15].

In a study by Meerwaldt et al. in patients on haemodialysis it was shown with a prototype of the AGE Reader that, after three years follow up, Skin AF was a strong independent predictor of overall and cardiovascular mortality, as were pre-existing cardiovascular disease, CRP and s-albumin. Skin AF was a stronger predictor of cardiovascular mortality than of overall mortality[5]. The prototype of the AGE Reader, the AF Reader (AFR) was validated by comparing Skin AF values with AGE content measured in extracts from skin biopsies of control and diabetic subjects[15], as well as from dialysis subjects[5]. In these studies the fluorescent AGE pentosidine, as well of the non-fluorescent AGEs carboxy(m)ethyllysine, CML and CEL, were significantly related to Skin AF. In a later combined analysis, Skin AF appeared to be related to 76% with dermal pentosidine[16]. Therefore, Skin AF is expected to measure mainly tissue-bound AGEs, leaving only a restricted influence for other variables.

Plasma AGE, low molecule weight AGE peptides in plasma, may be one of these remaining variables; plasma AGE show autofluorescence in the same range (ex: 370 nm, em:

440 nm) and have increased values in patients on haemodialysis, as compared to controls[3; 17]. These strongly increased values of the circulating AGE pentosidine and other AGE in haemodialysis patients are a consequence of the mentioned lack of removal[3; 18-21]. Henle et al. showed that plasma autofluorescence and plasma AGE levels were significantly decreased after haemodialysis[22]. Therefore, our hypothesis was that the response on haemodialysis can be used to investigate the influence of changes in plasma autofluorescence on Skin AF.

Objectives

The aim of the present interventional study was to investigate whether changes in Skin AF could be observed after a single haemodialysis session.

Material and Methods

Study design

A consecutive, prospective interventional study was performed at the haemodialysis centre at Norrlands University Hospital, Umeå. Measurements of Skin AF with an AGE Reader and sampling of blood plasma were performed before and after a dialysis session.

AGE Reader

Three measurements of Skin AF at slightly different skin sites on the forearm were performed at room temperature with the AGE Reader both before and after haemodialysis in a room with windows covered by curtains to avoid direct sun illumination (semi-dark environment). The AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands) illuminates a skin surface of ~ 4 cm², guarded against surrounding light, with a light source that mainly provides excitation light around a peak wavelength of 370 nm (range 350 - 420 nm). Reflected light from the skin and autofluorescence are measured simultaneously, using a built-in AvaSpec 2048 spectrometer (Avantes, Apeldoorn, The Netherlands) within the instrument. Skin AF was based on the ratio of the average light intensity per nanometre in the range between 420 and 600 nm and the average light intensity per nm in the range between 300 and 420 nm (AF in arbitrary units (AU)). Version 2.3 of the AGE Reader software has been used to work out the measurements. In this version, the effect of skin colour has been compensated for in the calculation of Skin AF[23].

Plasma Autofluorescence

Advanced glycation end products (AGE) were determined in fresh frozen plasma samples. Plasma samples were taken before and after dialysis according to a protocol of Schwedler et al.[20]. Plasma samples were 50 times diluted in phosphate buffered saline.

Plasma fluorescence, Plasma AF, was measured at 460 nm after excitation at 370 nm, using a FLUOstar Optima plate-reader (BMG Lab Technologies, Durham, USA).

Ethical considerations

All patients were informed and volunteered of free will. The local ethics committee approved the study.

Demography

This study included 35 patients, from which data of Skin AF and Plasma AF were available both before and after haemodialysis. The age range for the 26 men (67.6 ± 11.5) was not different from that of the nine women (59.6 ± 14.8). Both groups did not differ as well in their HD vintage (30.3 ± 18.4 months for men and 40.2 ± 34.2 months for women). Age adjusted comparison with control values was applied as well, using published results of AGE Reader measurements on healthy controls, giving the expected value of Skin AF for subjects with the same age as the patients in the present study[24].

The need for haemodialysis was caused by diabetic nephropathy for 7 patients (3 with diabetes mellitus type 1 and 4 with type 2), hypertension and/or reno-vascular disease for 8 patients, primary glomerular disorders for 8 patients, and by other reasons for the remaining 12 patients. Other demographic findings of the patients were 3/34 current tobacco users (9%, data of 1 missing) and 20/33 previous tobacco users (57%, data missing in 2), none with hepatitis B or C, eight with a previous myocardial infarction (23%), 5 with a previous stroke (14%), 18 with a history of cardiovascular disease (51%), 14 with diabetes mellitus (40%), 29 with hypertension (91%, data missing in 3) and three with a prior kidney transplant (9%). Median duration of haemodialysis was 22 months (range 1-106). Four patients were treated with the low flux filters FX10 (Fresenius, Bad Homburg, Germany) and one with Polyflux 17L (Gambro, Hechingen, Germany), while 30 were treated with high flux filters FX80 (n=15, Fresenius, Bad Homburg, Germany), Polyflux 140H (n=7) or Polyflux 210H (n=8, Gambro, Hechingen, Germany). Median dialysis time was 4 hours (3-5.5 hours). All patients were dialysed using 5 mmol/l glucose in the dialysate (Biosol A201.25 glucose 5 and Biosol A301.25 glucose 5), provided by Meda AB, Solna, Sweden. The dialysate had been sterilized by heat. All patients were offered a light meal during dialysis.

Statistical Analysis

For Skin AF, median values for each triple measurement were used. After using the Kolmogorov-Smirnov test, comparison between groups was performed with Student's T test for normal distributions, and Pearson's correlation coefficient was used. Paired statistical analysis was made by the paired Student's T test for normally distributed data.

SPSS statistical software (version 19.0; SPSS, Inc., Chicago, IL) was used for the analysis; a two-tailed p less than 0.05 was considered significant.

Results

Before dialysis, patients had Skin AF values that were 1.64 times higher than for age-matched healthy persons (p < 0.001), as shown in Figure 1, which is 65% higher on average.

Analyses of the whole group revealed that haemodialysis resulted in a non-significant reduction in Skin AF from 3.99 ± 0.7 to 3.88 ± 0.7 after dialysis (p = 0.19), as shown in Figure 2.
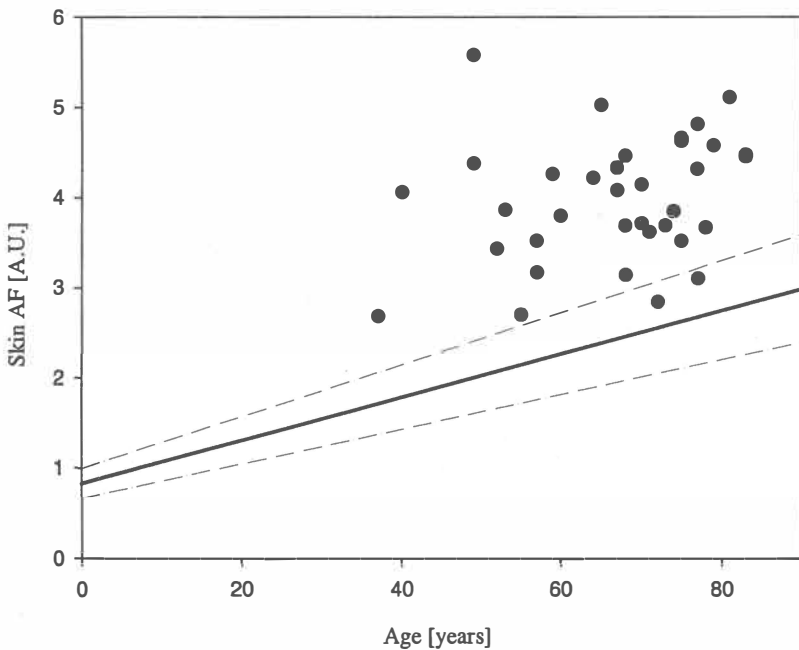
Fig. 1: Skin AF of each patient before haemodialysis as a function of age; the central line shows the mean value and the soft lines the standard deviation for the healthy age-matched persons (data for healthy persons derived from 24).

Plasma AF was reduced from 65262 ± 12398 AU (arbitrary units) before start of dialysis to 56438 ± 9049 after haemodialysis, which was a reduction of 14% (Figure 3, p < 0.001). The urea reduction during haemodialyses in these patients was at a median 70%.

No correlation was found between the decrease in Skin AF and the decrease in Plasma AF ($R^2 = 0.004$; p = 0.72).
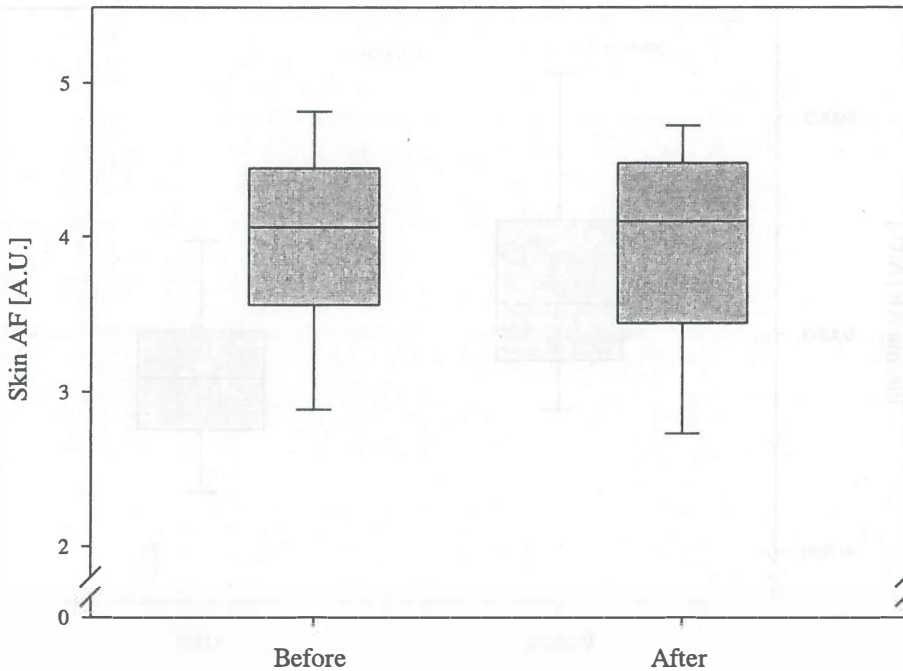
Fig. 2: Boxplot for Skin AF before and after haemodialysis. The paired test showed no significant difference (p = 0.19).

No effects of gender were observed in the reduction of Skin AF and Plasma AF. Women had significantly lower values of Skin AF than men, 3.5 ± 0.6 versus 4.2 ± 0.7 (p = 0.006) before haemodialysis, also after correction for age (p < 0.05), for which the results in healthy controls were used as given by the slope for control subjects in Figure 1. This lower level for women was also found after haemodialysis (p < 0.05).
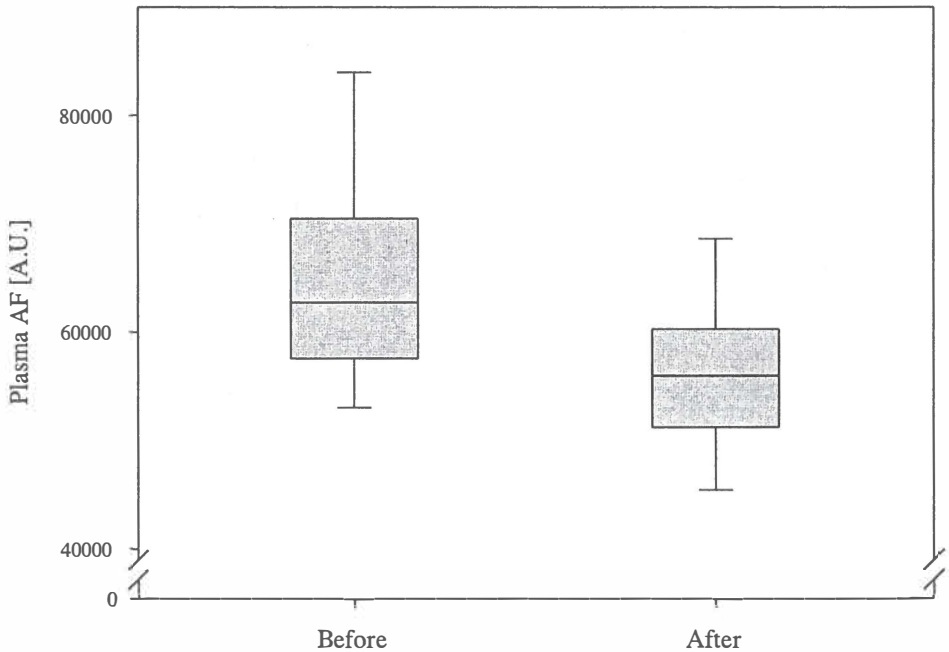
Fig. 3: Boxplot for Plasma AF before and after haemodialysis. The paired test showed a significant difference (p < 0.001).

Discussion

To our knowledge, this is the first intervention study that investigates the effect of haemodialysis on changes in Skin AF and Plasma AF in comparable wavelength ranges. The present study showed that the measured value of Skin AF was not significantly reduced by the significant changes of 14% in Plasma AF. It confirms that Skin AF is strongly correlated with the amount of skin AGE[16].

The strong correlation found between Skin AF and mortality by Meerwaldt et al.[5], indicates that Skin AF is mainly related to the more permanent accumulation of AGEs. Notable was that earlier results by Schwedler et al. and Busch et al.did not find a relation with mortality based on circulating (plasma) AGE in HD patients[20;21]. Also Nienhuis et al., studying patients with systemic lupus erythematosus, noted that the conclusions for circulating AGE differ from that for accumulated AGE values in tissue[25].

With respect to the plasma fluorophores in blood vessels it should be noted that they contribute to a much smaller extent to the measured autofluorescence at the skin surface as compared to fluorophores that have leaked out of these blood vessels. This is in agreement with the results of a study by De Jager et al, who studied the difference in response between patients with diabetes and controls in the first ten minutes after injection of sodium

fluorescein into the circulation[26]. It illustrates that a large part of the fluorophores remain hidden within the blood vessels because of strong light absorption by haemoglobin[27].

The present results show that the reduction in Plasma AF after HD does not lead to significant changes in Skin AF with the type of treatment used in this study. This indicates that the AGE present in the extravascular space is not significantly reduced either due to only a neglible amount of soluble AGE or due to an additional slight increase of AGE during HD e.g. by the food that the patients eat during the HD procedure.

The 14 % reduction of Plasma AF by haemodialysis is significantly less than the removal achieved of water soluble products, such as urea. Henle et al.[22] showed that high flux haemodialysis reduced fluorescent peaks present in plasma of about 30%. However, those data also showed a less than expected clearance of AGE products from plasma than if the products would have been totally unbound. This indicates that a larger part of the AGEs present in plasma are protein bound[28]. Therefore a removal will probably need prolonged and frequent sessions of standard haemodialysis to lower these compounds further. Another option would be the addition of adsorption devices to the haemodialysis system.

The present study showed that the skin autofluorescence level was significantly higher in haemodialysis patients compared to age matched healthy controls[24]. Such increased levels were also found in other groups of dialysis patients[5; 29-33].

Because Skin AF is a prominent marker for cardiovascular risk especially in haemodialysis patients[5], it is important to continue to investigate if any intervention may improve this variable. Further research should focus on the influence of variables such as dialysis modalities and food intake.

In conclusion, this study showed that Skin AF, a marker of CVD risk which is high in haemodialysis patients, was not significantly reduced after a single haemodialysis. Nevertheless, the reduction of AGE by adequate haemodialysis remains important from the point of view to minimize and even reverse the retention of solutes that contribute to the progression of cardiovascular morbidity in these patients.

Acknowledgments

Conflict of interest

R. Graaff is co-founder and stockholder of DiagnOptics Technologies B.V., the manufacturer of the AGE Reader. No economical support was provided by any company.

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Chapter 5

Increase in Skin Autofluorescence and release of Heart-type Fatty Acid Binding Protein in plasma Predicts Mortality of Hemodialysis Patients

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Abstract

Advanced Glycation End-products are uremic toxins that accumulate progressively in hemodialysis patients. The aim of this study was to assess the one year increase of Skin Autofluorescence (ΔAF), a measure of Advanced Glycation End-products accumulation and plasma markers, as predictors of mortality in hemodialysis patients.

169 Hemodialysis patients were enrolled in this study. Skin Autofluorescence was measured twice, one year apart using an AGE Reader. Besides routine blood chemistry, additional plasma markers including Superoxide Dismutase, Myeloperoxydase, Inter-Cellular Adhesion Molecule 1, C-Reactive Protein, Heart-type Fatty Acid Binding Protein, and von Willebrand Factor were measured at baseline. The mortality of hemodialysis patients was followed for 36 months.

Skin Autofluorescence values of the hemodialysis patients at the two time points were significantly higher ($p < 0.001$) than those of healthy subjects of the same age. Mean one year ΔAF of hemodialysis patients was 0.16 ± 0.06 which was around 7-9 fold higher than one year ΔAF in healthy subjects. Multivariate Cox regression showed that age, hypertension, one year ΔAF, C-Reactive Protein, Inter-Cellular Adhesion Molecule 1, and Heart-type Fatty Acid Binding Protein were independent predictors of overall mortality. Hypertension, one year ΔAF, C-Reactive Protein, and Heart-type Fatty Acid Binding Protein were also independent predictors of cardiovascular mortality.

One year ΔAF and plasma Heart-type Fatty Acid Binding Protein, used separately and in combination, are strong predictors of overall and cardiovascular mortality in hemodialysis patients.

Introduction

Hemodialysis (HD) patients in the USA have a 5-fold shorter life expectancy than healthy subjects of the same age; their average expected remaining lifetime is 6 years. The leading cause of death in patients with End-Stage Renal Disease (ESRD) is Cardiovascular Disease (CVD), and in the USA this is responsible for 40% of overall mortality[1].

Inflammation, endothelial dysfunction, protein-energy wasting, volume overload and oxidative stress are highly prevalent in HD patients and play an important role in the development of CVD in these patients[2]. Advanced Glycation End-products (AGEs) are uremic toxins that accumulate progressively in HD patients. AGEs levels are markedly increased in HD patients not only because of increased production by oxidative stress, but also because of impaired excretion[3]. AGEs cross-link with proteins of tissues with a slow protein turnover, such as collagen of the dermis and blood vessel walls. AGEs are considered to be biomarkers of long term increased inflammatory, oxidative and metabolic stress[1].

The measurement of Skin Autofluorescence (AF) is a non-invasive method to assess the level of AGEs that are present in tissue[2]. A single time point measurement of Skin AF has proven to be an independent predictor of cardiovascular mortality in HD patients[3]. It was shown that the Skin AF value in HD patients is higher than that of healthy subjects and that Skin AF correlates with HD vintage[4-6]. Therefore, it is expected that AGEs accumulation in the skin progresses rather rapidly in HD patients. However, to our knowledge, repeated measurements of Skin AF to assess the increase of Skin AF (ΔAF) over a period of time in HD patients have never been reported. We hypothesize that the measurement of the progression of AGEs accumulation in the skin may contribute to better understanding of the high mortality of HD patients.

The aim of this study was to assess in HD patients the one year ΔAF and various plasma markers, as predictors of overall and CVD mortality.

Materials and Methods

169 HD patients were enrolled in a clinical prospective study on Skin AF as a marker of AGE accumulation and mortality. Their Skin AF was measured and routine blood examinations were taken, as well as Skin AF and plasma markers of oxidative stress, inflammation, endothelial activation and cellular injury. The study protocol was approved by an Ethics Committee, and written informed consent was obtained from each patient. The following exclusion criteria were used: less than 3 months on HD; acute illness or hospitalization three weeks prior to the start of the study; neoplasm; previous kidney transplantation. The mortality of HD patients was followed for a period of 36 months after the first Skin AF measurement.

Dialysis Treatment

All patients underwent HD treatment three times per week with a median session duration of 4 hours. The duration of the HD session was individually adjusted, according to Kidney Disease Outcomes Quality Initiative guidelines, to maintain an equilibrated Kt/V >1.2. The patients were dialyzed with a non-glucose containing bicarbonate dialysis solutions (Aminal 100B + Aminal 100CK, Alkaloid, Skopje, Macedonia) using low-flux polysulfone (F10 HPS-surface area 2.2 m², Fresenius, Bad Homburg, Germany) or polyamide (Poliflux 6L-surface area 1.4 m², Gambro, Lund, Sweden) dialyzer membranes. The blood flow of the HD device was aimed to be at 300 ml/min and the dialysate flow was 500 ml/min. Patients received standard medical care as appropriate for HD patients.

Patient Characteristics

Hypertension was defined as a systolic blood pressure of >140 mmHg or a diastolic pressure of >90 mmHg measured on at least three different occasions. In addition, patients under antihypertensive medication were regarded as hypertensive patients.

Diabetes was defined by conventional American Diabetes Association criteria[7].

HD vintage was defined as the duration of the period between the initiation of long-term HD treatment and the start of the study.

Causes of death were classified as cardiovascular mortality (myocardial infarction, sudden death, stroke, and congestive heart failure) or non-cardiovascular death (neoplasm, infection etc.) according to The International Classification of Diseases coding criteria[8].

Advanced Glycation End-Products

Skin AF was measured using an AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands). The AGE-Reader illuminates a skin surface of approximately 4 cm² with excitation light mainly between 350 and 420 nm (peak excitation approximately 370 nm). The measured Skin AF is the average reflected light intensity between 420 - 600 nm, divided by the amount of reflected light between 300 - 420 nm, multiplied by 100, Skin AF = reflected light (420 - 600 nm) / reflected light (300 – 420 nm) * 100. Skin AF is measured in arbitrary unites (AU). The measurements were performed in triplicate at room temperature in a room with windows covered by curtains to avoid sun illumination (semi-dark). The median value of the triple measurement was used for further analysis. We used AGE Reader software version 2.3 that included correction for dark skin color[9].

Since the coefficient of variation for the AGE Reader has been addressed in several studies[3;10;11], in which it varies between 5 and 8% we used paired statistics and 169 patients to achieve an expected power of more than 80%. Paired statistics was also used to reduce the interference of confounding variables such as co-morbidities, life style and other demographic factors as many of them do not change in time. Skin AF was measured during

dialysis at two time points in the same HD patients: at the beginning of the study and after one year (median: 372 days, range: 370-374 days).

Biochemical Blood Analyses

Blood sampling for routine blood analysis was performed monthly. Additional blood samples were taken on the day of the first Skin AF measurement for determination of the markers of oxidative stress, inflammation, endothelial activation and heart and kidney injury. All blood samples (3 ml) were taken from the arterial line of the vascular access.

For each subject the routine monthly analysis that was performed closest to the day of first Skin AF measurement was used in our study. The following routine laboratory parameters were used: urea, creatinine, sodium (Na+), potassium (K+), calcium (Ca++), phosphates, hemoglobin concentration, platelets count, alkaline phosphates (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutathione-S-transferase (γ-GST), lactate dehydrogenase (LDH), plasma total proteins, plasma albumin, direct and total bilirubin, and uric acid.

Additional blood analysis of the concentration of superoxide dismutase (SOD), myeloperoxydase (MPO), inter-cellular adhesion molecule 1 (ICAM-1), c-reactive protein (hs-CRP), heart-type fatty acid binding protein (H-FABP) and von Willebrand factor (vWF) in blood plasma were performed. The methods used in the additional analysis have been previously described in detail[6].

Statistical Analyses

Comparisons between the normally distributed variables measured over time were performed with paired and group comparisons were made with independent Student t-test. If a non-normally distributed variable was involved in the comparison then non-parametric methods were used. Cox regression and Kaplan-Meier curves were used to determine the influence of AGEs accumulation and other variables on the overall mortality of HD patients. In the multiple Cox regression models we used only the variables that had $p < 0.10$ in the univariate analysis. SPSS statistical software (version 16.0 SPSS, Inc., Chicago, IL) was used; two-tailed $p < 0.05$ was considered significant. Data are shown as mean ± standard deviation, unless specified otherwise.

Results

The mean age of the 169 HD patients at the start of this study was 56 years (range 23 - 82). The mean period on HD (HD vintage) was 8.7 years (range 0.4 - 27.9). The weekly average dialysis time was 12.3 hours (range 9 - 15). Diabetes was present in 41 (24%) HD patients. The characteristics of all HD patients (Table 1) and diabetic HD patients (Table 2) are listed separately. In the time period of 36 months of follow-up 49 HD patients died (29

%): 32 from CVD, 15 from non-CVD (infections, liver failure etc.) and 2 from unknown causes. During the follow-up period 4 patients received a kidney transplant and 2 patients moved to other HD centers.

Table 1. Demographic and clinical parameters of the all hemodialysis patients at the start of the study

Variable	All HD Patients (N = 169)
Gender (Male)	104 (61%)
Age (years)	56 ± 13
Smokers	17 (10%)
Consumed Alcohol	24 (14%)
Body Mass Index (kg/m²)	23.2 ± 4.8
CVD	31 (18%)
Diabetes	41 (24%)
Hypertension	34 (18%)
HbSAg	19 (11%)
HD vintage (years)	8.71 ± 6.3
Average weekly duration of the HD treatment (hours)	12.3 ± 0.86

CVD-Cardiovascular Disease; HbSAg-Hepatitis B Surface Antigen; HD-Hemodialysis

Table 2. Demographic and clinical parameters of the diabetic hemodialysis patients at the start of the study

Variable	Diabetic HD Patients (N = 41)
Gender (Male)	27 (66%)
Age (years)	60 ± 11
Smokers	3 (7%)
Consumed Alcohol	7 (17%)
Body Mass Index (kg/m²)	23.8 ± 5.0
CVD	12 (29 %)
Hypertension	10 (24%)
HbSAg	7 (17%)
HD vintage (years)	5.87 ± 4.6
Average weekly duration of the HD treatment (hours)	12.3 ± 0.83

CVD-Cardiovascular Disease; HbSAg-Hepatitis B Surface Antigen; HD-Hemodialysis

Skin Autofluorescence and Plasma Markers

Skin AF values of the HD patients at the two time points were significantly higher (p < 0.001) than those of healthy subjects of the same age from our previous study[6]. In the univariate analysis at baseline, diabetic HD patients demonstrated elevated Skin AF values as compared to non-diabetic patients (3.49 ± 0.98 versus 3.20 ± 0.87 AU; p < 0.05). Furthermore, Skin AF at baseline correlated with age (R = 0.28; p < 0.05) and HD vintage (R = 0.32; p < 0.05). Using the variables that had a $p \leq 0.10$ in the univariate analysis, the independent markers of Skin AF at the start of the study appeared to be diabetes, age and HD vintage.

Using a paired t-test we found a significant increase of Skin AF in the measurements, which were one year apart: (3.24 ± 0.87 vs. 3.39 ± 0.76; p = 0.03) as shown in Figure 1. The mean one year ΔAF (AF2 - AF1) was 0.16 ± 0.06. In the univariate analysis the one year ΔAF correlated with HD vintage (R = 0.22; p < 0.05) and the level of MPO (R = 0.25; p < 0.05). Diabetic HD patients had borderline higher one year ΔAF than non-diabetic HD patients (0.14 ± 0.55 vs. 0.25 ± 0.55 AU; p = 0.097). In the multiple linear regression analysis we included HD vintage, MPO plus the presence of CVD morbidity and diabetes, which showed p < 0.10 in the univariate analysis. We found that MPO at the start of the study was the sole independent predictor of the one year ΔAF.

Skin Autofluoresence in Hemodialysis Patients
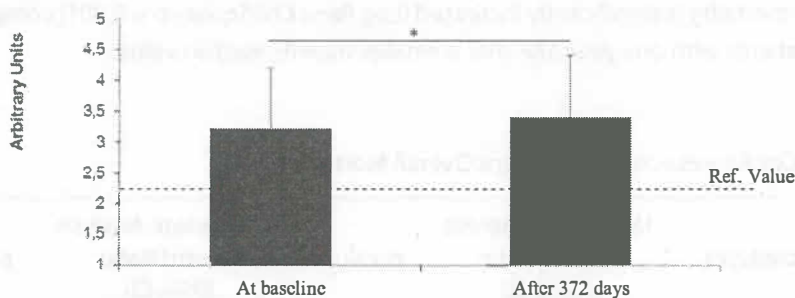
Figure 1. The Skin AF of the HD patients at baseline and after 372 days and in healthy controls. The Skin AF of HD patients at the two time points was significantly (p < 0.001) higher than in the healthy controls of the same age from our previous study which was 2.2 arbitrary unites, the reference line ([6]). * p < 0.05 paired t-test.

The levels of the additional plasma markers are given in Table 3. SOD, MPO, ICAM-1, hs-CRP and H-FABP were significantly higher than their reference values for healthy subjects, except vWF that was within the reference range.

Table 3. Additional Blood Analysis at start of the study

Parameter	Reference Value	Value
SOD (ng/ml)	$48 \pm 2\,9$[27]	540 ± 410
MPO (ng/ml)	43.8 ± 23.3[28]	594 ± 290
ICAM-1 (ng/ml)	0.111 ± 0.017[29]	2.12 ± 0.88
hs-CRP (µg/ml)	<5	12.6 ± 42
H-FABP (ng/ml)	4.4 ± 3.9[30]	60.7 ± 19
vWF (% pool)	55-200	123 ± 57

SOD-superoxide dismutase; MPO-myeloperoxydase; ICAM-1 inter-cellular adhesion molecule 1; hs-CRP-c-reactive protein; HFABP-heart-type fatty acid binding protein; vWF-von Willebrand factor

Patient's Overall and Cardiovascular Mortality

In the univariate Cox regression analysis of overall and CVD mortality we used all clinical, demographical and biochemical parameters and Skin AF. In the univariate Cox regression analysis of overall and CVD mortality, diabetes and several other parameters were found to be significant. In the multivariate Cox regression analyses with a cut off of $p < 0.05$ all the variables were included that had a $p \leq 0.10$ in the univariate analysis. In those analyses diabetes was included, but it was neither found to be an independent marker of overall nor of CVD mortality. In the multiple Cox regression analysis of overall mortality the age, hypertensicn, one year ΔAF, hs-CRP, ICAM-1 and H-FABP (given with their hazard ratios and confidence intervals in Table 4) appeared to be the independent predictors. Figure 2 shows that if one year ΔAF of a HD patient is larger than the median value, the likelihood of overall mortality is significantly increased (Log Rank Chi-Square $p = 0.001$) compared to the HD patients with one year ΔAF that is smaller than its median value.

Table 4. Cox Regression of HD Patient Overall Mortality

Characteristics	Univariate Analysis Hazard Ratio (95% CI)	p-value	Multivariate Analysis Hazard Ratio (95% CI)	p-value
Age	1.05 (1.02-1.07)	**<0.001**	1.04 (1.00-1.08)	**0.04**
Male	1.76 (0.95-1.07)	0.07		
CVD History	2.90 (1.60-5.28)	**<0.01**		
Diabetes	2.73 (1.55-4.82)	**<0.001**		
Hypertension	2.35 (1.31-4.22)	**<0.001**	2.59 (1.13-5.93)	**0.02**
HbSAg	2.95 (1.47-5.92)	**<0.01**		
HD Vintage	0.95 (0.91-1.00)	0.06		

Annual ΔAF	2.01 (1.10-3.67)	**0.02**	2.52 (1.35-4.71)	**<0.001**
hs-CRP	1.01 (1.01-1.02)	**<0.001**	1.02 (1.00-1.03)	**0.010**
ICAM-1	1.70 (1.36-2.15)	**<0.001**	1.97 (1.26-3.07)	**<0.001**
SOD	1.66 (1.01-2.72)	**0.04**		
MPO	1.00 (1.00-1.00)	**<0.001**		
H-FABP	1.01 (1.01-1.02)	0.10	1.02 (1.00-1.03)	**0.03**
Albumin	0.93 (0.87-0.98)	**0.006**		

CVD-Cardiovascular Disease; HbSAg-Hepatitis B Surface Antigen; HD-Hemodialysis; AF-Autofluorescence; hs-CRP-C-Reactive Protein; ICAM-1-Intercellular Adhesion Molecule-1; SOD-Superoxide Dismutase; MPO-Myeloperoxidase; H-FABP-Heart type-Fatty acid binding protein.
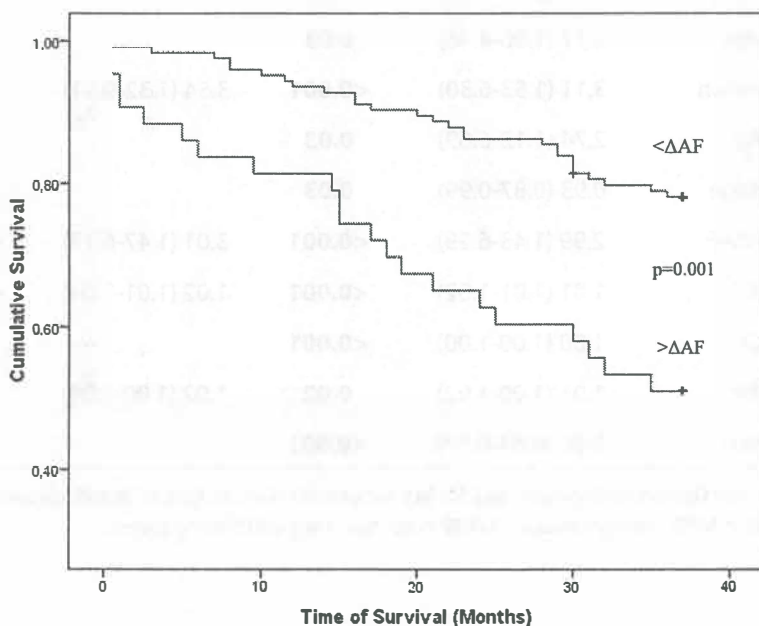

Figure 2. The overall mortality of hemodialysis (HD) patient as a function of the one year increase of Skin Autofluorescence; The HD patients that had higher than median one year increase of Skin Autofluorescence had significantly (p = 0.001) higher overall mortality compared to HD patients that had lower than median one year increase of Skin Autofluorescence; >ΔAF: above median one year increase of Skin Autofluorescence, <ΔAF: below median one year increase of Skin Autofluorescence.

In the multivariate Cox regression analysis of CVD mortality the independent predictors were hypertension, one year ΔAF, hs-CRP and H-FABP (Table 5). Figure 3 shows that if H-FABP of HD patients is larger than the median value, the likelihood of CVD mortality

is significantly increased (Log Rank Chi-Square p=0.02) compared to HD patients with H-FABP smaller than its median value.

Table 5. Cox Regression of HD Patient Cardiovascular Mortality

Characteristics	Univariate Analysis Hazard Ratio (95% CI)	p-value	Multivariate Analysis Hazard Ratio (95% CI)	p-value
Age	1.05 (1.02-1.08)	**<0.001**		
Male	2.44 (1.05-5.64)	**0.04**		
CVD History	2.33 (1.07-5.42)	**0.03**		
Diabetes	2.17 (1.06-4.46)	**0.03**		
Hypertension	3.11 (1.53-6.30)	**<0.001**	3.54 (1.32-9.51)	**0.02**
HbSAg	2.74 (1.12-6.69)	**0.03**		
HD Vintage	0.93 (0.87-0.99)	**0.03**		
Annual ΔAF	2.99 (1.43-6.29)	**<0.001**	3.01 (1.47-6.17)	**<0.001**
hs-CRP	1.01 (1.01-1.02)	**<0.001**	1.02 (1.01-1.04)	**<0.001**
MPO	1.00 (1.00-1.00)	**<0.001**		
H-FABP	1.01 (1.00-1.02)	**0.02**	1.02 (1.00-1.04)	**<0.001**
Albumin	0.90 (0.84-0.97)	**<0.001**		

CVD-Cardiovascular Disease; HbSAg-Hepatitis B Surface Antigen; HD-Hemodialysis; AF-Autofluorescence; hs-CRP-C-Reactive Protein; MPO-Myeloperoxidase; H-FABP-Heart type-Fatty acid binding protein.
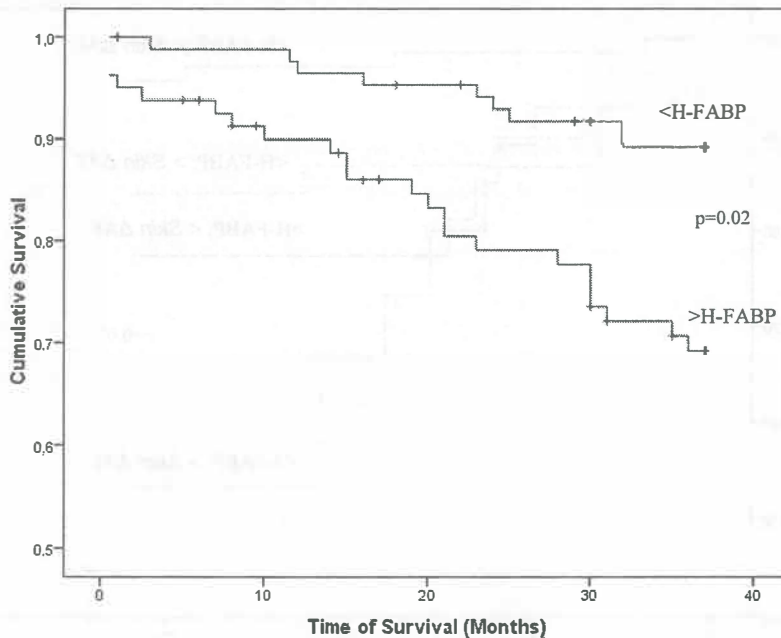
Figure 3. The cardiovascular mortality of hemodialysis (HD) patient as a function of the Heart-type Fatty Binding Protein; The HD patients that had higher than median Heart-type Fatty Binding Protein had significantly (p=0.02) higher cardiovascular mortality compared to HD patients that had lower than median Heart-type Fatty Binding Protein; >H-FABP: above median Heart-type Fatty Binding Protein, <H-FABP: below median Heart-type Fatty Binding Protein.

The present results showed that higher values of H-FABP and one year ΔAF indicate higher risk of overall and cardiovascular mortality. The combined use of one year ΔAF and H-FABP as predictors of the cardiovascular mortality was tested. The four groups with different combination of above and below median values of H-FABP and one year Skin ΔAF were createsd. The mortality rates of the groups were significantly (p = 0.01) different. The group of HD patients with values of H-FABP and Skin ΔAF below their medians had the lowest cardiovascular mortality, whereas the group with values of H-FABP and Skin ΔAF above their medians had highest cardiovascular morality. The cardiovascular mortality of the two other groups was in between. In addition to the separate use the combined use of one year ΔAF and H-FABP as predictors of the cardiovascular mortality risk is also possible as shown by the Kaplan-Meier curves in Figure 4.
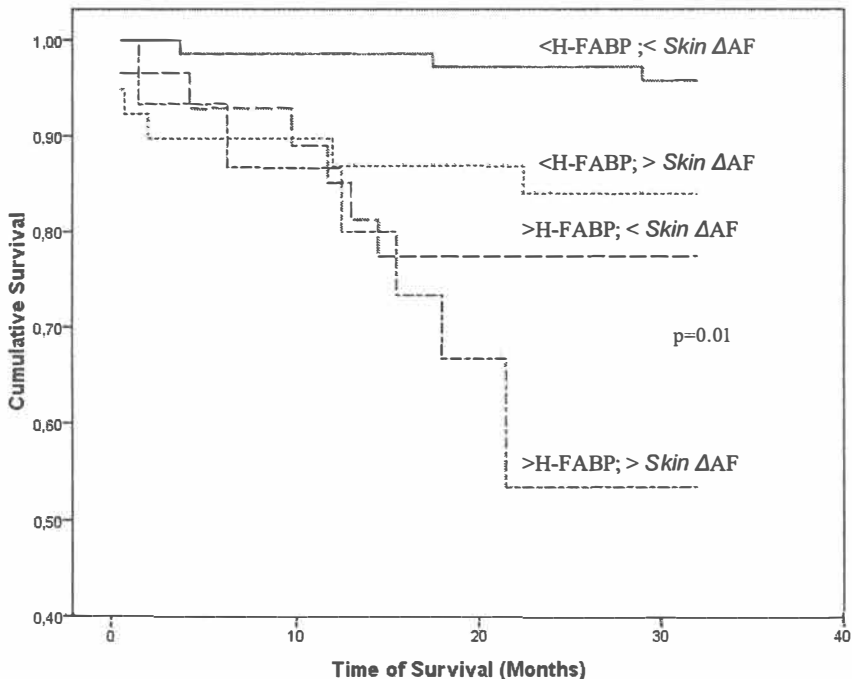
Figure 4. Cardiovascular mortality of Hemodialysis patient (HD) as a function of Heart-type Fatty Acid Binding Protein (H-FABP) and one year increase of Skin Autofluorescence (Skin ΔAF). In addition to the separate use the combined use of one year ΔAF and H-FABP as predictors of the cardiovascular mortality risk is possible. ; >H-FABP: above median Heart-type Fatty Binding Protein, <H-FABP: below median Heart-type Fatty Binding Protein.; >ΔAF: above median one year increase of Skin Autofluorescence, <ΔAF: below median one year increase of Skin Autofluorescence.

Discussion

Our study shows for the first time that in HD patients the rate of one year ΔAF and H-FABP are predictors of overall and CVD mortality. As far as we know this is the first study that analyzes the progression of AGE accumulation in HD patients over a year, with a follow up on mortality for 36 months. The mean one year ΔAF of 0.16 AU was in line with the results from our previous retrospective baseline study[6], where we estimated the average increase of Skin AF in HD patients with CVD to be in the range from 0.21 to 0.34 AU per year depending on the presence or absence of diabetes. The HD patients in our study had a 7-9 fold higher one year ΔAF than healthy subjects, which showed an increase of 0.017-0.023 AU per year, as found previously[6;9]. The sole independent predictor of one year ΔAF in our study was MPO, an oxidative stress marker. This indicates that the major contributor of one year ΔAF is the level of oxidative stress in the HD patients.

In the present study we found that at baseline the level of Skin AF is influenced by the presence of age, diabetes and HD vintage. The relationship between the age of HD patients and Skin AF was already known. Our data also confirmed the connection between Skin AF and the presence of diabetes in HD patients[3;6]. Furthermore, the level of Skin AF of diabetic HD patients is higher than the level of Skin AF in prevalent diabetic patients with similar age[12]. This indicates that ESRD is a separate contributor of AGEs accumulation in these patients. Additional proof for this is the correlation between Skin AF and HD vintage, a finding that was also published before[3;6].

The higher one year ΔAF indicated an increased rate of AGEs accumulation in the skin of HD patients than in healthy subjects, probably as a result of the increased oxidative stress, inflammation, endothelial activation and heart injury, either due to the renal failure and/or to the HD treatment itself. This assumption is strengthened by the elevated levels of the measured plasma markers of oxidative stress (SOD and MPO), inflammation (hs-CRP), endothelial activation (ICAM-1) and cellular injury (H-FABP) found in this study. Impaired excretion of AGE free adducts and AGE peptides during hemodialysis may also have contributed.

The mortality rate (overall and CVD) in the follow-up period was comparable to the mortality rates of other reports[13]. Compared to other studies, the novel finding is that the one year ΔAF can predict mortality of HD patients. Meerwaldt et al. found that a single point Skin AF measurement was a good predictor of mortality in HD[3] and diabetic[12] patients. Diabetic patients have a higher risk for increased AGE accumulation and mortality than healthy persons[12]. Nevertheless, the presence of diabetes in the studied group of HD patients did not discriminate an increased hazard in the multivariate Cox Regression analyses; previous studies[3;14] also came to the same conclusion. This may be due to the fact that the dialysis procedure interferes much more with the morbidity of the patients and the effect of diabetes is only marginal. In the present study, the one year ΔAF proved to be a significant independent predictor of overall and CVD mortality regardless of the presence or absence of diabetes.

H-FABP was an independent predictor of overall but especially of CVD mortality in HD patients. Others demonstrated that H-FABP is a strong predictor of mortality in patients with cardiovascular disorders; acute pulmonary embolism[15], chronic pulmonary hypertension[16], acute coronary syndrome[17;18], acute chest pain[19] and chronic heart failure[20]. H-FABP is a very sensitive marker of acute myocardial[21;22;23] and kidney damage[24;25], and is now known also to contribute to a more accurate prediction of the three-years-survival of HD patients maybe because the HD treatment itself caused myocardial damage[26].

Finally, our study did not only find that one year ΔAF and plasma H-FABP are independent predictors of overall and cardiovascular mortality in HD patients. In addition to the separate use the combined use of one year ΔAF and H-FABP as predictors of the cardiovascular mortality risk is also possible. The reason for this can be that one year ΔAF

and H-FABP are the result of two different phenomena, oxidative stress and organ damage, respectively.

Conclusions

One year ΔAF and plasma H-FABP, used separately and in combination, proved to be strong predictors of overall and CVD mortality in HD patients.

Limitations

It should be noted that a large part of AGEs present in the human body are non-fluorescent. However, previous results showed that Skin AF may function as a marker of the AGE pool, based on the strong correlations with both fluorescent and non-fluorescent skin AGE levels measured by conventional biochemical means in skin biopsies of HD and diabetes patients[2;3]. Another limitation of our study is that we cannot exclude the influence of fluorophores other than AGEs on Skin AF measurements. Despite major improvements that have been made in measuring Skin AF in persons with dark skin, still no measurements are possible in persons with skin reflectance <6% (approximately Fitzpatick type VI skin color, the darkest skin color type). In our study group we had a very small number of darker skin patients.

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Chapter 6

Does Hepatitis C Increase the Accumulation of Advanced Glycation End-Products in Hemodialysis Patients?

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Abstract

Background: Hepatitis C may cause increased levels of oxidative stress that contribute to accumulation of Advanced Glycation End-Products (AGEs), which increase the risk of cardiovascular disease (CVD). The aim of this study was to determine the influence of Hepatitis C on AGE accumulation in hemodialysis patients.

Methods: AGEs accumulation was measured by means of Skin Autofluorescence (AF) in 92 hemodialysis (HD) patients and 93 age-matched healthy controls. In the HD patients CVD-related biochemical variables were also measured. The HD patients were tested for Hepatitis C Virus (HCV) antibodies and allocated to a HCV+ or HCV- group.

Results: Skin AF of the healthy subjects was lower than skin AF in the HD patients (3.13±0.95 vs. 2.2±0.47; p<0.001). We calculated the average increase of skin AF in the healthy subjects to be 0.017 AU per year, being 14 times lower than in HD patients with CVD only and 20 times lower than in HD patients suffering from combined CVD and Diabetes Mellitus (DM). Multivariate regression analysis showed that AGEs accumulation in HD patients can be described by the independent effects of age, DM, CVD and HD vintage. Although ICAM-1 and liver enzymes were elevated in HCV+ HD patients, levels of oxidative stress markers and skin AF were not significantly different between HCV+ and HCV- HD patients.

Conclusions: AGEs accumulation was higher in the HD patients than in the healthy controls. AGEs accumulation did not differ in HCV+ and HCV– HD patients. This might be due to the fact that hepatitis C did not cause oxidative stress in our HD population. Independent markers of AGEs accumulation were age, HD vintage, DM and CVD, but not hepatitis C.

Introduction

The close interaction between renal and cardiovascular function makes patients who suffer from chronic renal disease 10 to 20 times more susceptible for developing cardio-vascular disease (CVD) in comparison to a healthy population[1,2]. Hemodialysis (HD) treatment of End Stage Renal Disease patients (ESRD) even increases this risk, for example by the HD related blood-material interactions, hemodynamic instabilities and initiation of anemia. Because of the high prevalence of CVD in HD patients (± 40%), CVD has become the major cause of hospitalization of HD patients, thus reducing the life expectancy of these patients dramatically[3]. High prevalence and high incidence of cardiovascular mortality in HD patients are of multi-factorial origin. Disturbances in the balance between oxidants and antioxidants, the carbohydrate and lipid metabolism, the immuno-inflammatory system and malnutrition are all thought to play a major role in a cascade of organ dysfunction resulting in patient death[4].

Another complicating factor in the HD treatment is the high prevalence of hepatitis C virus (HCV) infection among the HD population. It is estimated that the world-wide prevalence of HCV is around 3 % or 200 million people[5], whereas the percentage of HCV+ patients in the HD population is much higher than in the general population and can vary from 5-25 % in USA and Western Europe, up to 80 % in the middle East [6]. The association between the prevalence of HCV and overall mortality of HD patients has been proven by others[7-9]. In a meta-analysis study HD patients with HCV appeared to have an adjusted relative risk of all-cause mortality of 1.34 (1.13-1.59)[10]. Apart from this finding it should be noted that the relation between CVD and HCV in HD patients is still not fully established, as some researchers had contradictory findings[11-14].

AGEs are formed during glycemic and oxidative stress, with increased levels in Diabetes mellitus (DM), renal failure and chronic inflammatory conditions[15]. AGE accumulation affects the blood vessels and is connected with the development of CVD[16]. Skin Autofluorescence (AF) is a non-invasive method to estimate the AGE accumulation in the body[17] , which has shown to be a strong non-invasive CVD risk marker in HD patients[18] and has therefore been used in the present study. The aim of this study was to determine the influence of hepatitis C on AGE accumulation and other cardiovascular risk parameters in HD patients. Furthermore we assessed other factors that possibly influenced AGEs accumulation in our patient population.

Subject and Methods

Ninety-two patients with ESRD were enrolled in a clinical cross-sectional study on AGE accumulation and CVD risk assessment in HD patients. The study protocol was approved by an Ethics Committee, and written informed consent was obtained from each patient. The following exclusion criteria were used: less than 3 months on HD; acute illness or

hospitalization three weeks prior to the start of the study; neoplasm; kidney transplantation.

The patients were assigned to one of the two groups: HCV+ and HCV-. The determining criterion for the presence of hepatitis C were the anti-hepatitis C virus antibodies measured by an ELISA Ortho HCV 3.0 SAVe kit (Ortho Diagnostics, Amersham, UK) as part of the routine blood analysis of the HD patients done on 6-weeks intervals. Both groups were matched as well for age, sex and dialysis vintage.

Ninety-three age-matched healthy control subjects were also enrolled in this study. The controls were selected from a group of people that visited a general practitioners' office in the same area. The inclusion criteria were: the control subjects had an overall ASA (American Society of Anesthesiologists)[19] physical condition classification score of 1 or 2. Controls should have been registered at that general practitioner for more than a year.

In the healthy controls the age, gender, smoking habits past and present and body-mass index (BMI) were recorded, and skin AF was measured.

Dialysis Treatment

All patients underwent hemodialysis (HD) treatment three times per week with a median session duration of 4 hours. The duration of the HD session was individually adjusted, according to Kidney Disease Outcomes Quality Initiative guidelines[20], to maintain an equilibrated Kt/V >1.2. The patients were dialyzed with non-glucose containing bicarbonate dialysis solutions and low-flux polysulfone (Fresenius, Bad Homburg, Germany) or polyamide (Gambro, Stockholm, Sweden) dialyzer membranes. The device blood flow was 300 ml/min and the dialysate flow was 500 ml/min. Patients received standard medical care as appropriate for HD patients.

Patient Characteristics

CVD, hypertension and Diabetes were diagnosed by independent specialists, using the following criteria: A patient was considered to have CVD when a history of coronary heart disease, peripheral vascular disease, or cerebro-vascular disease was present, (*International Classification of Diseases, Tenth Revision, Clinical Modification* codes I20, I21, I63, I70, and I73) [21].

Hypertension was defined as a systolic blood pressure of >140 mmHg or a diastolic pressure of >90 mmHg measured on at least three different occasions[22]. Also patients under antihypertensive medication were regarded as hypertensive patients. Finally, when nephrosclerosis was present we also considered hypertension to be the cause of ESRD.

Diabetes was defined by conventional American Diabetes Association criteria[23].

HD vintage was defined as the period between the initiation of long-term HD treatment and the time of the measurement.

Skin Autofluorescence

Accumulation of skin AGEs was estimated from skin autofluorescence (AF) that was measured using the AGE-Reader (DiagnOptics Technologies BV, Groningen, The Netherlands) which was manufactured on the basis of the previously described prototype[18]. The AGE Reader has an improved spectrophotometer and a better available UV lamp, which has less excitation below 350 nm. Briefly, the AGE-Reader illuminates a skin surface of approximately 4 cm² with excitation light mainly between 350 and 420 nm (peak excitation approximately 370 nm). The measured skin AF is the average light intensity between 420-600 nm, divided by that between 300-420 nm, multiplied by 100. Skin AF is measured in arbitrary units (AU). The measurements were performed in triplicate at room temperature in a semi-dark environment.

Skin AF was measured in the HD patients during dialysis. In a group of 24 HD patients skin AF was measured before and after dialysis. Finally, Skin AF was also measured in 'the control subjects.

Biochemical Blood Analysis

The sampling of routine blood analysis was done monthly before and after the HD session. Additional blood samples were taken at the end of the HD session, on the day of the skin AF measurements, for determination of the oxidative stress, inflammation, endothelial activation and organ damage. In order to investigate the effect of the HD treatment on the level of the measured markers, extra blood samples were taken from 24 patients before the same HD session. Albumin levels before and after the HD treatment were measured in order to correct for the possible hemoconcentration after the HD treatment. All blood samples (3 ml) were taken from the vascular access.

The following routine laboratory parameters were used in our study: hemoglobin concentration; platelets count; alkaline phosphates (AP); aspartate aminotransferase (AST); alanine aminotransferase (ALT); gamma glutathione-S-transferase (γ-GST); lactate dehydrogenase (LDH); plasma total proteins ;plasma albumin; direct and total bilirubin; hepatitis-B-virus surface antigen (HBsAg) and uric acid.

The concentration of Superoxide dismutase (SOD), Myeloperoxidase (MPO), Inter-Cellular Adhesion Molecule 1 (ICAM-1), C-reactive protein (CRP), and Heart-type Fatty Acid Binding Protein (H-FABP) in blood plasma was measured, using the following methods:

- *Superoxide Dismutase (SOD),* which catalyzes the dismutation of the superoxide anion (O2⁻) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity in plasma, the Water soluble tetrazolium 1 microtiter plate method[24] was used.

- *Myeloperoxidase (MPO),* is a leukocyte-derived enzyme that catalyzes the formation of a reactive oxidant species like hypochlorous acid (HOCl) and tyrosyl. Increased level of MPO is a sign of increased oxidative stress. MPO was measured by ELISA (Hytest, Turku, Finland).
- *C-reactive Protein (CRP)* is an acute phase plasma protein produced by the liver and adipocytes. CRP is mainly used as a marker of inflammation. We used a high sensitivity CRP ELISA (Dakopatts, Glostrup, Denmark).
- *Inter-Cellular Adhesion Molecule 1*(ICAM-1) is a cell adhesion molecule. ICAM-1 is a ligand for the Lymphocyte Function-associated Antigen-1 (LFA-1), a part of the leukocyte integrin family of molecules. When leukocytes are activated they bind increasingly to endothelial cells via ICAM-1/LFA-1 and then transmigrate into the tissue. Plasma concentrations of ICAM-1 are indicative of endothelial activation. ICAM was measured by ELISA (R&D Systems Minneapolis ,USA)
- *Heart-type Fatty Acid Binding Protein* (H-FABP) is a protein with a size of 15 kDa. H-FABP is abundantly expressed in cardiomyocytes, but also in distal tubular cells of the kidney and skeletal muscle. H-FABP is used as a marker of kidney and heart injury. H-FABP was measured by ELISA (Hytest, Turku, Finland)

Statistical Analyses

Comparisons between the four groups were performed with Student t-test. The skew-distributed variables were log transformed. The correlations were analyzed with Spearman rank method. Multivariate regression analyses were performed for determination of independent relationships between the risk markers and the presence of CVD and, AGE accumulation and its potential causes. SPSS statistical software (version 14.0 SPSS, Inc., Chicago, IL) was used for the analysis; two-tailed $P < 0.05$ was considered significant. Data are shown as mean ± standard deviation.

Results

The 92 patients treated by maintenance hemodialysis were divided into two groups according to their anti HCV antibody status: an HCV+ group of 48 patients and an HCV- group consisting of 44 patients. The age of all studied patients ranged from 21 to 86, with a mean of 58. The mean HD vintage was 4.28 years (range 0.25-8.92). The weekly average session of all patients was 12.2 hours. 12 (13%) Were active smokers. The patient characteristics are listed in Table 1.

Table 1. Demographic and clinical parameters of the hemodialysis patients enrolled in the
study

Variable	N=93
Gender (Male)	58 (61%)
Age (years)	58 ± 13
Smokers Current	12 (13%)
Diabetic	32 (35%)
CVD	23 (25%)
Average Weekly Duration of HD session (hours)	12.2 ± 0.62
Hemodialysis Vintage (years)	4.28 ± 2.49
Hypertension as a cause of ESRD	26 (28%)
Anti-HCV (+)	48 (52%)
HbSAg (+)	13 (14%)

ESRD= End-Stage Renal Disease; HbSAg= Hepatitis B Surface Antigen; HCV = Hepatitis C Virus

The 93 age-matched controls subjects had an average age of 54±17 years (vs. 56±13
for the HD patients; p=0.83), 31 (33%) were smokers, 62 non-smoker from which 20 were
ex-smokers, the average smoking vintage of combined current and ex-smokers was 20±12
years. The controls characteristics are listed in Table 2.

Table 2. Demographic parameters and Skin Autofluorescence results of the healthy
subjects.

Variable	N=93
Gender (Male)	48 (52%)
Age (years)	54±17
Smokers (Current/Ex/Never)	31/20/42 (33/21/45%)
Average Smoking Vintage-Current and Ex (Years)	20±12
Amount of Cigarettes (per day)	21±9
BMI (kg/m2)	27.8±18
Skin AF (AU)	2.2±0.47

AF-Autofluorescence; BMI-Body Mass Index;

HCV Status and Routine Biochemical Parameters

The prevalence of CVD (p=0.01) and Diabetes (p<0.001) reduced with the increase of the HD vintage, whereas the HCV prevalence increased (p< 0,001). Table 3 shows the results for the patients of both groups. The mean blood pressure was lower (114±33 vs. 133±35 mmHg, p=0.02) in the HCV+ HD group. Although the routine biochemical parameters hemoglobin, alkaline phosphatase, alanine and aspartate amino-transferase, direct bilirubin, LDH, γ-GST, plasma globulins and uric acid were increased in the HCV+ HD patients, all measured values remained within their reference ranges.

The multiple regression analysis showed that HCV+ had direct effect on the following diagnostic parameters (given with their odds ratios and confidence intervals in brackets): blood pressure 0.973 (0.951-0.995, p=0.016), bilirubin direct 3.062 (1.03-9.13, p= 0.045), LDH 1.02 (1.01-1.03, p=0.001), log(γGST) 12.84 (1.53-107.44, p=0.019) and globinemia 1.23 (1.03-1.46, p=0.023).

Skin Autofluorescence

Skin AF in the HD patients was higher than in age-matched controls (3.13±0.95 vs. 2.20±0.47 AU; p<0.001). There was no significant difference between skin AF values of HCV+ and HCV- HD patients (3.20±0.96 vs. 3.03±0.96 AU; p=0.39) as shown in Figure 1. In the control group Skin AF correlated strongly with age (R=0.55; p<0.001) and smoking vintage combined for current and ex-smokers (R=0.50; p<0.001). In the HD patients Skin AF correlated with age (R=0.24; p<0.05) and with HD vintage (R=0.22; p< 0.05). Diabetic HD patients had increased Skin AF compared to the non-diabetic (3.48±0.95 versus 2.93±0.90 AU; p=0.01) and patients with hypertension as a cause of ESRD had higher Skin AF than HD patients with other cause of ESRD (3.28 ± 0.92 versus 2.81 ± 0.99 AU; p < 0.02).

Table 3. Clinical, demographic and biochemical parameters of HCV+ and HCV- hemodialysis patients

Variable	Reference Values	HCV+ 48 (52%)	HCV- 44 (48%)	p-Value
Demographic and Clinical Parameters				
Age (years)		56±13	59±13	0.255
Sex-males		28 (58%)	28 (64%)	0.672
Avg.Weekly duration of the Hemodialysis treatment (hours)		12.3±0.8	12.1±0.3	**0.092**
Hemodialysis Vintage (years)		4.6±2.4	4.0±2.5	0.260
Hypertension as a cause of ESRD		13 (27%)	13 (30%)	0.820
Mean Blood Pressure (mmHg)	75-135	114±33	133±35	**0.020**
Diabetes Mellitus		19 (40%)	13 (30%)	0.383
CVD		11 (23%)	12 (27%)	0.810
Biochemical Parameters *Routine Blood Analysis*				
HbSAg (+)		7 (15%)	6 (14%)	0.999
Hemoglobin (g/L)	120-180	116±13	109±12	**0.028**
Ferritin (mg/L)	0-300	436±261	496±263	0.450
Platelet count (10^9/L)	140-340	201±84	208±76	0.701
Log Alkaline Phosphatase (U/L)	1.57-2.10	2.10±0.27	1.85±0.2	**0.001**
Log AST (U/L)	1-1.53	1.36±0.25	1.17±0.16	**0.001**
Log ALT (U/L)	1-1.66	1.44±0.31	1.24±0.21	**0.001**
Bilirubin Total (mmol/L)	6.8-20.5	7.25±1.68	7.0±2.4	0.588
Bilirubin Direct (mmol/L)	1.5-6.8	3.41±0.75	3.05±0.69	**0.037**
LDH(U/L)	213-423	281±82	198±71	**0.001**
Log γ-GST (U/L)	0.96-1.81	1.62±0.37	1.20±0.38	**0.001**
Plasma Proteins (g/L)	63-83	71.5±8.7	68±5.2	**0.039**
Plasma Albumin (g/L)	35-50	36.5±5.0	36.3±5.1	0.905
Plasma Globulins (g/L)	27-35	35.7±7.2	31.6±5	**0.006**
Uric Acid (mmol/L)	143-446	434±76	385±82	**0.013**
Additional Blood Analysis				
Skin AF (AU)	2.10±0.45	3.20±0.96	3.03±0.96	0.391
Log SOD (ng/ml)	1.68±0.21 (unlogged 48±2)[34]	2.59±0.22	2.68±0.25	**0.067**
MPO (ng/ml)	43.80± 23.30[35]	674±311	598±248	0.213
Log CRP (µg/ml)	<0.69 (unlogged <5)	0.55±0.98	0.74±0.97	0.262
ICAM-1 (ng/ml)	0.111± 0.017[36]	2.20±0.81	1.84±0.62	**0.022**
H-FABP (ng/ml)	4.4±3.9[37]	36.7±21.8	36.5±28.7	0.975

AF-Autofluorescence; ALT-Alanine aminotransferase; AST-Aspartate aminotransferase; CRP-C Reactive Protein; CVD-Cardiovascular disease; ESRD-End Stage Renal Disease; GST-glutathione S-transferase; HbSAg-Hepatitis B Surface Antigen; H-FABP Heart-type fatty acid-binding protein; ICAM-1-Intercellular Adhesion Molecule-1; LDH-Lactate Dehydrogenase; MPO-Myeloperoxidase; SOD-Superoxide Dismutase
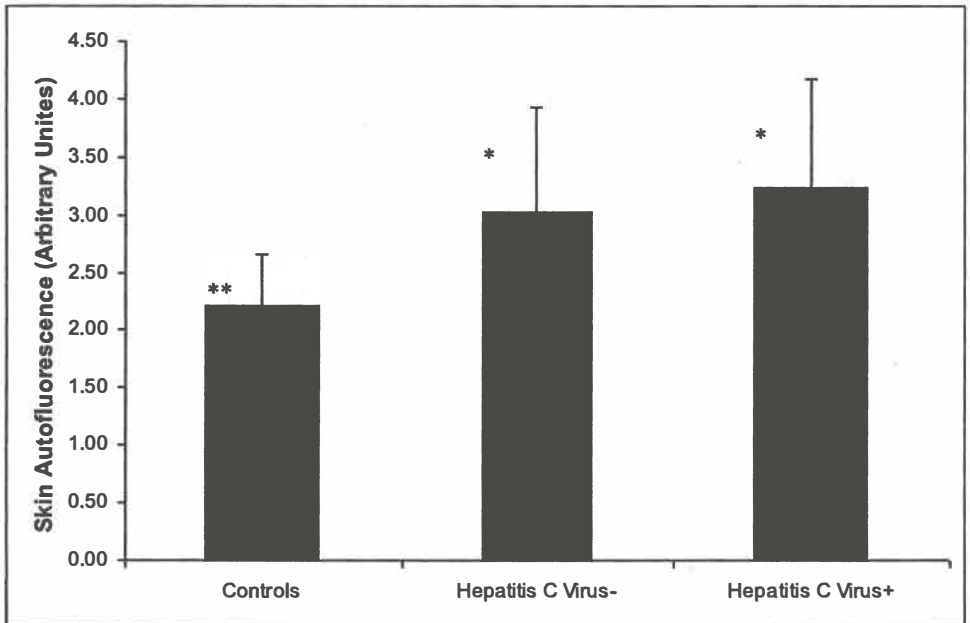
Figure 1. The Skin Autofluorescence in age-matched controls and hepatitis C+ and hepatitis C-hemodialysis patients. The skin autofluorescence is significantly lower in the controls compared with the hemodialysis patients in both groups * p<0.001.

Multivariate regression showed that AGE accumulation in HD patients could be described by the independent effects of age (p<0.05), diabetes (p<0.01) and HD vintage (p=0.03) with R^2=0.23), whereas in the healthy subjects Skin AF could be described by age (p<0.001) and smoking vintage (p<0.001; R^2=0.61).

From the results of the control group, we fitted Skin AF from healthy subjects as a function of age (R=0.55; p=0.01). To calculate the additional Skin AF value of the HD subjects, we subtracted the values obtained from the fit of the controls as a function of age from the real measured value of a HD subject. The additional Skin AF increase correlated with HD vintage only in CVD patients with diabetes and showed to be 0.34 AU per year for the diabetes patients (R = 0.70; p<0.01) as shown in Figure 2. A significant trend of 0.021 AU per year for CVD patients without DM (R=0.54; p=0.05) was calculated. For the non-CVD patients no significant trend line was obtained. Furthermore, we calculated the average increase of Skin AF in healthy subjects to be 0.017AU per year that was 14 times lower than in HD patients with CVD only and 20 times lower than in HD patients with CVD and DM.
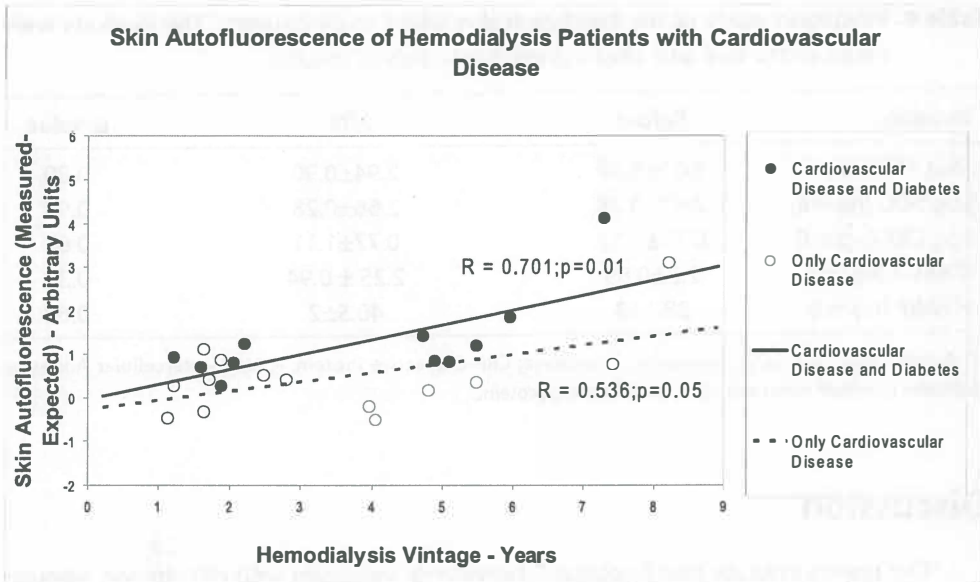
Figure 2. The Skin Autofluorescence difference between healthy controls and hemodialysis patients with cardiovascular disease of the same age, given according to the duration of HD;

Additional Biochemical Markers

The measured values of SOD, MPO, ICAM-1, CRP and H-FABP were higher than the normal reference values listed in Table 3. Log SOD was near significantly lower in the HCV+ HD patients compared with HCV- (2.59±0.22 vs. 2.68±0.25 ng/ml; p=0.067) whereas ICAM-1 was higher (2.20±0.81 vs. 1.84±0.62 ng/ml; p=0.022). SOD, MPO and ICAM-1 were also included in the multiple regression analysis, but did not appear to be independent markers of the presence of the HCV infection. Also no significant differences were found between the HCV+ and HCV- patients with regard to the CVD markers.

We questioned whether the used plasma markers were substantially affected by the proceeding dialysis procedure itself. Therefore SOD, ICAM-1, CRP and H-FABP were measured before and after a single dialysis session. The MPO molecule is too large (140 kD) to pass the dialysis membrane. No significant changes in SOD, CRP, ICAM-1 and H-FABP were measured in these samples (see table 4).

Table 4. Validation study of the biochemical markers in 24 patients. The markers were measured before and after a single hemodialysis session.

Variable	Before	After	p-value
Skin AF (A.U.)	3.01±0.89	2.94±0.90	0.89
Log SOD (ng/ml)	2.67±0.28	2.66±0.28	0.95
Log CRP (µg/ml)	0.73±1.12	0.77±1.11	0.67
ICAM-1 (ng/ml)	2.2±0.89	2.25 ± 0.94	0.31
H FABP (ng/ml)	38.1±3	40.5±2	0.68

AF-Autofluorescence; SOD-Superoxide Dismutase; CRP-C Reactive Protein; ICAM-1-Intercellular Adhesion Molecule-1; H-FABP Heart-type fatty acid-binding protein.

Discussion

Our results indicate that hepatitis C prevalence increased with HD vintage whereas diabetes and CVD prevalence dropped at the same time. Most likely the inverse association between HCV+ and the prevalence of CVD and diabetes was due of the high early mortality of CVD and diabetes HD patients. This higher mortality was also illustrated by the AGE accumulation per year in HD CVD patients, which was 14-20 folds higher than in the healthy subjects.

The AGE accumulation was higher in the HD patients than in the healthy controls. The level of AGE accumulation in HD patients was mostly due to their age, HD Vintage and diabetes, whereas in the healthy subjects it was due to their age and smoking vintage. Although the level of enzymatic activity and ICAM were higher in HCV+ than in HCV- patients, the AGEs accumulation did not differ between the HCV+ and HCV– patients.

Skin AF, a marker of tissue AGE accumulation, was much higher in HD patients compared to the age-matched controls. Skin AF and AGE accumulation are markers of glycemic and oxidative stress or of reduced clearance and have been found to be independent, strong predictors of CV mortality in diabetes and renal failure. A comparison of Skin AF in healthy subjects and HD patients was for the first time done by Meerwaldt et al.[18] , who found 2.5 fold higher Skin AF in HD patients whereas J. Hartog et al.[25] and T.Matsumoto et al.[26] found 1.5 times higher values of Skin AF, which is similar to our study. In all of these studies[18;25;26] Skin AF in HD patients was strongly correlated with age and HD vintage as in our study. We confirmed the observation of Meerwaldt et al[18] that Skin AF correlates with the presence of diabetes. Moreover, we found that the AGE accumulation per year in HD patients was 14 to 20 times higher than in healthy subjects depending of the presence of CVD and diabetes.

HCV+ HD patients presented higher enzymatic activity, but all elevated parameters were within reference value range except for ICAM-1. This is line with the finding of others on the progress of the HCV infection in HD patients. Okuda K et al.[27] compared the progress

of Hepatitis C in HD patients with non-uremic controls over a period between 4 and 23 years. During the first 4 years 25 % of the controls developed cirrhosis whereas the HD patients did not. Also, it is interesting that Okuda et al.[27] found that all of the patients that were followed for more than 15 years had asymptomatic HCV infection. These results were confirmed by our study as all the HCV+ patients that were more than 9 years on HD were having asymptomatic HCV infections. This means that the HCV infection is not a strong factor for development of a liver dysfunction in patients that were longer on HD.

The most important factor for the AGE accumulation is the level of the oxidative stress for which we used the SOD and MPO as major markers. Akiyama et al. concluded that SOD is up-regulated and can thus be used as a marker of increased oxidative stress, especially when leucocytes are activated, like during HD treatment by the membrane[28]. The findings of other studies on the oxidative stress in HCV+ HD subject are controversial. There are studies that found that the HCV infection in HD patients is responsible for increased[29;30] oxidative stress, whereas others suggest that HCV infection can be even protective[31]. However, those studies were either underpowered or did not measure oxidative stress markers directly. We found nearly significant reduction in the level of SOD and no changes of MPO in the HCV+ HD patients which indicated the low influence of HCV on oxidative stress. The AGE accumulation in our study reflected the oxidative stress over a long period of time and agreed with those results, as it did not show differences between HCV+ and HCV- HD patients either. To our knowledge, we were the first to investigate the influence of hepatitis C on AGE accumulation in HD patients. The studies that investigated the influence of HCV on circulatory AGEs in non-HD patients, did not show differences between healthy subjects and HCV+ patients[32;33] as well. Regarding HD patients, Nascimento et al.[30] found higher levels of plasma pentosidine in HCV+ compared to HCV- HD patients. In contrast, we did not find higher AGE accumulation in HCV+ compared to HCV- HD patients.

Limitations of our study are that we cannot exclude the influence of uremic toxins or skin fluorophores other than AGEs on skin AF measurements. Furthermore, it should be noted that most of AGEs present in the human body are nonfluorescent. However, previous results showed that skin AF may function as a marker of the AGE pool, based on the strong correlations with both fluorescent and nonfluorescent skin AGE levels measured by conventional biochemical means in skin biopsies of HD and diabetes patients[17;18]

Our result of SOD, MPO and Skin AF measurement clearly showed that the oxidative stress was not higher in the HCV+ HD patients. Additional research on the influence of HCV on oxidative stress in HD patients is needed due to the conflicting results between various studies.

In conclusion, AGE accumulation is higher in HD patients than in healthy subjects, but does not differ between HCV+ and HCV- hemodialysis patients. This is probably a result of the similar level of oxidative stress between the HCV+ and HCV- patients. The major predictors of the AGE accumulation are: the HD treatment itself, diabetes, CVD and the age of the patient. HCV does not influence AGE accumulation in HD patients.

Acknowledgments

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Chapter 7

The influence of body mass index on the accumulation of advanced glycation end-products in hemodialysis patients

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Abstract

Background and Aims: The level of Skin Autofluoresence (AF) at a given moment is an independent predictor of mortality in Hemodialysis (HD) patients. Skin AF is a measure of the accumulation of Advanced Glycation End-Products (AGEs). The aim of the study was to estimate the influence of nutrition on the one year increase of (ΔAF) in HD patients.

Methods and Results: 156 HD patients were enrolled in this study. Skin AF, Body Mass Index (BMI), Superoxide Dismutase, Myeloperoxydase, C-Reactive Protein, Inter-Cellular Adhesion Molecule-1, von Willebrand Factor and Heart-type Fatty Acid Binding Protein were measured four times at intervals of approximately half a year. The data from the monthly routine blood analysis were also used. Daily calorie, protein and AGEs intake were assessed from food recordings over period of one week.

A U-shaped relation was found between baseline BMI and ΔAF. The lowest point of the U-shaped curve is 24.3 kg/m^2. In the univariate analysis of the contributors to the one year ΔAF we found that beside (BMI - 24.3)^2, AGE and calorie intake as well as Myeloperoxydase and HD vintage were significant. The multivariate analysis the sole independent predictor of the one year ΔAF was (BMI - 24.3)^2.

Conclusions: It appears that calorie, protein and AGE intake hardly influence the one year ΔAF in HD patients. However, BMI is a predictor of the one year ΔAF which is a strong predictor of mortality of HD patients. The BMI of HD patients of around 24 kg/m2 results in the lowest one year ΔAF.

Introduction

Advanced Glycation End-products (AGE) are a result of non-enzymatic glycoxidative reactions also known as Maillard reactions. AGE content of food can vary significantly[1]. Prolonged administration of an AGE-rich diet in rodents resulted in higher level of circulatory AGE, decreased renal function[2], diabetic complications[3], endothelial injury[4] and shortened life-span[5]. Several of these effects were also reported to occur in obese subjects[6] and in patients with diabetes[7]. However, some controversy remains on the relationship between AGE intake and plasma AGE in humans[8-11].

Reduced excretion of free AGE, AGE free adducts and AGE peptides in renal failure, and especially in End Stage Renal Disease (ESRD), may make this condition a human model in which the effects of higher AGE supply from food becomes important in enhancing AGE accumulation[12]. Uribarri et al[13] first showed that AGE intake correlates with circulating AGE levels in renal failure patients.

Autofluorescence (AF) measurement is a non-invasive technique which can be used to assess the level of AGE in the skin[14]. The level of Skin AF at a given moment has shown to be an independent predictor of the mortality in Hemodialysis (HD) patients[15]. Recent results showed that one year increase of Skin AF (ΔAF) is even stronger predictor of mortality of HD patients than a single point measurement of Skin AF[16]. A single point measurement of Skin AF did not correlate with the AGE intake of elderly healthy subjects[17]. A close interaction exists between AGE, protein and calorie intake and Body Mass Index (BMI)[1]. However, to our knowledge no prospective study to estimate influence of AGE intake and BMI on the accumulation of skin AGE (one year ΔAF) in HD patients has ever been performed.

The aim of this study was to investigate the influence of nutrition and BMI on the rate of AGE accumulation of HD patients.

Methods

In the study population Skin AF was measured at four timepoints with approximately a six month interval, the total duration of the study was 19 months. Apart of routine blood analysis, also blood samples were taken to assess markers of oxidative stress, inflammation, endothelial activation and myocardial damage. Furthermore, patients were asked to record their daily food intake over a period of a week. From these recordings daily calorie, protein and AGEs intake were calculated. This procedure was performed in autumn and was repeated after 6 months in spring to find out whether their daily food intake changed during the seasons. The study protocol was approved by the hospitals Ethics Committee, and written informed consent was obtained from each patient. From a parent population of 169 prevalent HD patients at the Department of Nephrology in Skopje using exclusion criteria we have enrolled 156 HD patients in this prospective study. Our exclusion criteria

were: less than 3 months on HD; acute illness or hospitalization three weeks prior to the start of the study; neoplasm; previous kidney transplantation.

Dialysis Treatment

All patients underwent HD treatment three times per week with a median session duration of 4 hours. The duration of the HD session was individually adjusted, according to Kidney Disease Outcomes Quality Initiative guidelines, to maintain an equilibrated Kt/V >1.2. The patients were dialyzed with a non-glucose containing bicarbonate dialysis solutions (Aminal 100B + Aminal 100CK, Alkaloid, Skopje, Macedonia) and low-flux polysulfone (F10 HPS-surface area 2.2 m², Fresenius, Bad Homburg, Germany) or polyamide (Poliflux 6L-surface area 1.4 m², Gambro, Lund, Sweden) dialyzer membranes. The blood flow of the HD device aimed to be at 300 ml/min and the dialysate flow was 500 ml/min. Patients received standard medical care as appropriate for HD patients.

Patient Characteristics

Hypertension was defined as a systolic blood pressure of > 140 mmHg or a diastolic pressure of > 90 mmHg measured on at least three different occasions.

Diabetes was defined by conventional American Diabetes Association criteria[18].

HD vintage was defined as the duration of the period between the initiation of long-term HD treatment and the start of the study.

Data Collection

Biochemical Blood Analyses

The routine monthly blood analysis that was performed closest to the day of Skin AF measurements were used in our study. Additional blood analyses were also performed. Oxidative stress markers Superoxide Dismutase (SOD) and Myeloperoxydase (MPO), inflammation marker C-Reactive Protein (hs-CRP), endothelial activation markers von Willebrand Factor (vWF) and Inter-Cellular Adhesion Molecule-1 (ICAM-1), and myocardial and kidney damage marker Heart-type Fatty Acid Binding Protein (H-FABP) were measured. The methods used in the additional analysis have been previously described in detail[19]. The blood samples for the additional blood analyses were taken on the day of the Skin AF measurements.

Nutritional Records

The two dietary records, one in autumn and one in spring, from the HD patients were obtained to assess the calorie, protein and AGE intake per day and to investigate the existence of possible seasonal variations. Patients were given instructions how to record their food intake and to estimate the portion sizes. It was emphasized to record the day and time when meals, snacks and beverages were taken, a description of food, drinks, methods of preparations, missed meals; amounts consumed in restaurants and the amounts of consumed processed foods. The dietary questionnaire was used to calculate the AGEs intake using the food content of εN-carboxymethyllysine as common AGEs marker, according to Goldberg et al.[1]. We adapted Goldberg`s food content tables for local traditional meals using the closest analog of a certain local traditional meal. The amount of calories and proteins was calculated using tables from The United States Department of Agriculture Nutrition's Database[20].

BMI was calculated as a ratio of dry end-dialysis weight (in kilograms) to squared height (in meters)[21].

Advanced Glycation End Products Measurements

Skin AF was measured using an AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands). This device has been previously described in detail[14]. Briefly, the AGE-Reader illuminates a skin surface of approximately 4 cm² with excitation light mainly between 350 and 420 nm (peak excitation approximately 370 nm). The measured skin AF is the average reflected light intensity between 420-600 nm, divided by the amount of reflected light between 300-420 nm, multiplied by 100. Skin AF was measured in Arbitrary Unites (AU) using software version 2.3 that included correction for skin color[22]. All measurements were performed in triplicate on the forearm on slightly different locations at room temperature in a room with windows cowered by curtains to avoid sun illumination. Skin AF was measured in the HD patients during dialysis at 4 time-points: at the beginning of the study and after 6, 13 and 19 months. The first and third time points were in winter and the second and fourth were in summer.

Statistical Analyses

Comparisons between the variables were performed with paired or independent Student t-test depending of the relation between the variables. If the data were not normally distributed a $\log_{10}$ transformation was used to obtain a normal distribution. The correlations were analyzed with the Spearman rank method. In order to find a non-linear correlation between the variables first we checked if there is a correlation between the variable of interest and another U-shape transformed variable using the equation y = [variable - variable(median)]². Then, if this correlation was significant using SPSS we obtained the best

fitting values for the equation $y = a$ [variable $- b]^2 + c$, where a is a factor, b represents the value of the variable that corresponds to the lowest point of the U-shaped curve, and c is the minimum. Multiple linear regression analysis was used to find the independent factors contributing to AGE accumulation . Unless otherwise specified data are shown as mean ± standard deviation.

Results

The age of the 156 HD patients was 56 years (range 19 - 84). From them 97 (62%) were male, 31 (20%) had CVD, and 37 (24%) had diabetes. The HD vintage of these patients was 8.7 years (range 0.4 - 28). The patients' characteristics are given in Table 1. During the 19 months of follow-up, 25 HD patients died (16 %). During the follow-up period 3 patients received a kidney transplant and 2 patients moved to other HD centers. Thus at 30 patients not all four Skin AF measurements were available however if two measurement one year apart were available than they were included in the analysis if not they were excluded.

Table 1. The characteristics of all 156 HD patients at the beginning of the study.

Variable	Reference Value	Value
Age (years)		56 ± 13
Gender (Male)		97 (62%)
Smokers		12 (8%)
CVD		31 (20%)
Diabetes		37 (24%)
Hypertension		33 (21%)
HD vintage (years)		8.7 ± 6.6
Average weekly duration of the HD treatment (hours)		4.1 ± 0.2
Body Mass Index (kg/m²)	18-25	23.6 ± 4.8
SOD (ng/ml)	48 ± 2[45]	540 ± 410
MPO (ng/ml)	43.80 ± 23.30[46]	594 ± 290
hs-CRP (µg/ml)	<5	12.6 ± 42
ICAM-1(ng/ml)	0.111 ± 0.017[47]	2.12 ± 0.88
vWF (%pool)	50 – 166%[48]	123 ± 57
H-FABP (ng/ml)	4.4 ± 3.9[49]	60.7 ± 19

HD-Hemodialysis; SOD- Superoxide Dismutase; MPO- Myeloperoxydase; ICAM-1- Inter-Cellular Adhesion Molecule 1; CRP- C-Reactive Protein; H-FABP- Heart-type Fatty Acid Binding Protein; vWF- von Willebrand Factor;

Skin Autofluorescence, Nutrition and Biochemical Analysis

The daily AGE intake in autumn, 9.34 ± 4.01 MU/day did not significantly differ from its value in spring, 9.89 ± 4.33 MU/day. The protein intakes in autumn and spring were 1.27 ± 0.69 and 1.31 ± 0.60 g/kg of body weight per day respectively, which was around the upper limit of the KDOQI guidelines which recommend a protein intake of 1.2 to 1.3 g/kg of body weight per day[23]. Furthermore, the calorie intakes of 30.4 ± 9.4 and 32.4 ± 7.9 Kcal/kg of body weight per day, in autumn and in spring respectively, were also within KDOQI guidelines of 30 - 35 Kcal/kg of body weight per day for HD patients below 60 years of age [23]. The AGE intakes as well as the protein and calorie intakes were not significantly different between autumn and spring records. The protein, calorie and AGE intake correlated with each other (R = 0.56 p < 0.01; R = 0.36 p < 0.01; R = 0.33 p < 0.01).

The level of SOD, MPO, hs-CRP, ICAM-1 and H-FABP at all timepoints were significantly higher than the reference values for healthy subjects. The values of the additional blood analysis at start of the study are shown in Table 1.

The BMI at the start of the study was 23.6 kg/m^2 (range 14.3 - 47.1). Twenty-three (11.6 %) HD patients had a BMI < 18.5 kg/m^2 and were considered to be underweight whereas 39 (19.6 %) had BMI > 25 kg/m^2 and were considered overweight according to WHO[21]. Using a paired t-test we found that the BMI of the HD patients increased significantly during the course of the study (BMI$_1$ vs. BMI$_4$; 23.6 ± 2.8 vs. 24.2 ± 3.0; p = 0.03). The mean one year increase of BMI (BMI$_3$ - BMI$_1$ or BMI$_4$ - BMI$_2$; in case both were available an average was used) was 0.31 with a standard error of 0.11 kg/m^2.

Using a paired t-test we found a significant ΔAF (ΔAF) in the measurements that were one year apart: AF$_1$ vs. AF$_3$ (3.24 ± 0.87 vs. 3.39 ± 0.76; p = 0.03), and AF$_2$ vs. AF$_4$ (3.43 ± 0.83 vs. 3.57 ± 0.88; p = 0.02). The combined one year ΔAF of both years, defined as the difference between the time points that were 12 months apart (AF$_3$ - AF$_1$ or AF$_4$ - AF2; in case both were available an average was used) was 0.15 AU with a standard error of 0.09.

We investigated the presence of linear and non-linear relationships between BMI and nutrition, one year ΔAF and additional blood analysis parameters, because non-linear relationships between BMI and various variables were found in previous studies[24]. We found a linear relation between BMI and AGE, protein and calorie intake and one year increase of MPO (R=0.239 p=0.01; R=0.239 p=0.01; R=456 p=0.01; R=0.228 p=0.03). A non-linear U-shaped relation was found between BMI and one year ΔAF. We fitted a U-shaped curve that describes the non-linear relationship between BMI and one year ΔAF, as shown in Figure 1, where the U-shaped curve showed to have its lowest point at a BMI of 24.3 kg/m^2.

Table 2. Linear and Non-Linear Correlation Between BMI and daily food intake, Skin Autofluorescence and Additional Blood Analysis

Variable	BMI		(BMI-24.3)²	
	R-value	p-value	R-value	p-Value
AGE Intake per day	R = 0.255	p = 0.003		NS
Protein per day per body weight	R = 0.226	p = 0.008		NS
Calories per day per body weight	R = 0.465	p < 0.001		NS
Annual ΔAF		NS	R=0.236	p=0.012
Annual ΔSOD		NS		NS
Annual ΔMPO	R = 0.182	p = 0.043		NS
Annual ΔCRP		NS		NS
Annual ΔICAM-1		NS		NS
Annual ΔvWF		NS		NS
Annual ΔH-FABP		NS		NS

AGE- Advanced Glycation End-products; AF- Autofluorescence; SOD-Superoxide Dismutase; MPO-Myeloperoxidase; CRP- C-Reactive Protein; ICAM-1 Intercellular Adhesion Molecule-1; vWF-von Willebrand Factor; H-FABP- Hearth type Fatty Binding Acid Protein; NS non-significant
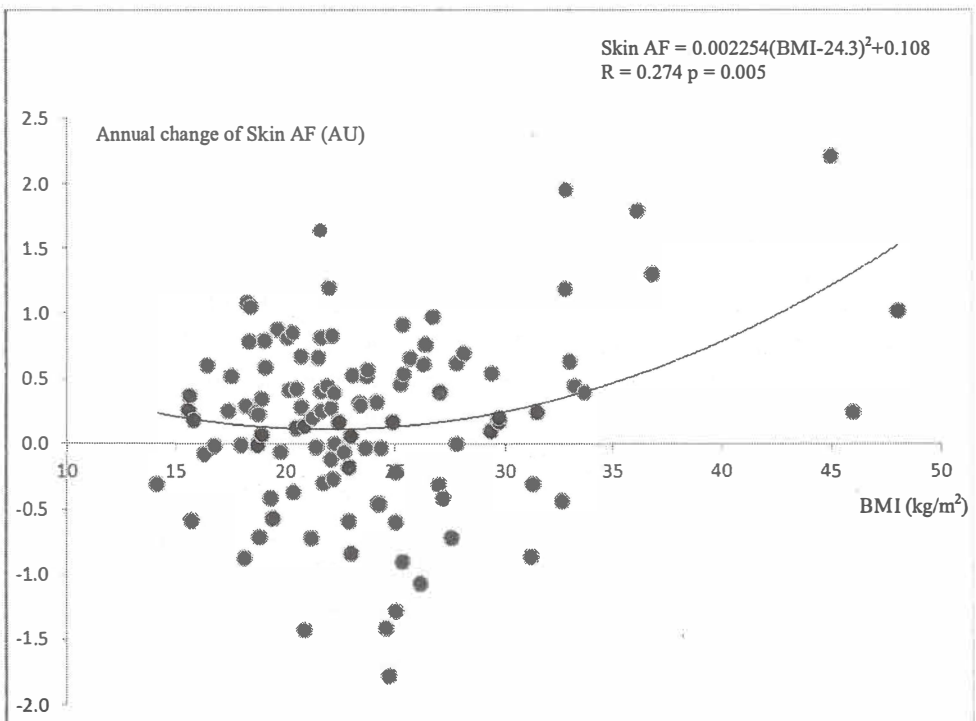

Skin AF = 0.002254(BMI-24.3)²+0.108
R = 0.274 p = 0.005

Figure 1. Non-linear relation between annual change of Skin Autofluorescence (AF) and Body Mass Index (BMI)

In the univariate analysis of the contributors to the one year ΔAF we found that beside $(BMI - 24.3)^2$, AGE and calorie intake as well as MPO and HD vintage were significant. In the multivariate analysis of the contributors to the one year ΔAF all the variables that had a p < 0.10 in the univariate analysis were used, plus the presence of CVD morbidity and diabetes at baseline. The sole independent predictor of the one year ΔAF was $(BMI - 24.3)^2$ as shown in Table 2.

Discussion

AGE, caloric and protein and intake do not influence the one year ΔAF in HD patients. BMI is in a non-linear way a predictor of the one year ΔAF with the lowest increase at a BMI of around 24 kg/m2.

The daily intake of AGE of the HD patients did not differ significantly between autumn and spring, with a mean AGE intake of 9.62 MU/day. This is 40% lower when compared to the AGE intake of HD patients in the United States who were reported to have an average AGE intake of 16 MU/day[13]. Vlassara et al[11] and Negrean et al[25] described prospective studies using a low AGE diet of 3 - 4 MU/day or a high AGE diet of 15 - 16 MU/day. In this regard the daily AGE intake of our study group was intermediate to those studies.

The mean intake of proteins and calories per day correlated with the mean AGE intake per day. The reason for this was that the most energy-rich food contained the highest concentration of AGE per gram. Also protein rich food has a high AGE content, whereas the food that was low in AGE content was also low in protein content[1]. This was also the reason why the protein, calorie and AGE intake had a correlation with the BMI of the HD patients during the study. The correlation between nutrition and BMI is expected and well known.

In the present study Skin AF of the HD patients at all timepoints was higher than Skin AF of healthy volunteers of the same age[19], as reported earlier by several groups[15;26;27]. The results of the one year ΔAF of HD patients in this study were similar to the results of a previous study which used only 2 time points to measure the one year ΔAF[16]. Earlier studies found that a single measurement of Skin AF had a positive correlation with BMI in healthy subjects[28] whereas BMI and Skin AF did not correlate hemodialysis patients[15]. In addition to these findings, the present study found a non-linear relationship between BMI and one year ΔAF.

Another important contributor for the AGE accumulation is the level of oxidative stress for which we used SOD and MPO as biochemical markers. Akiyama et al.[29] concluded that SOD is up-regulated in HD patients, especially because of leucocyte activation by the dialysis membrane and thus can be used as a marker of increased oxidative stress. This is in line with our findings; the measured values of SOD and MPO at all timepoints were higher than reference values for control subjects, indicating that the level of oxidative stress was increased in our HD population.

The mortality rate of HD patients during this study was lower than the average mortality rate in Europe according to the ERA-EDTA registry[30] due to still underdeveloped renal transplantation program in R.Macedonia.

We also assessed the relationship between the ΔAF in time and the AGE intake. The one year ΔAF had a borderline negative correlation with AGE and calorie intake. Previously, Jochemsen et al[17] investigated the relationship between nutrition including AGE intake and Skin AF. They did not manage to find a relationship between the AGE intake and the Skin AF in elderly healthy subjects at a given moment. In their study Skin AF correlated negatively with the calorie intake, a finding that is in line with our results. In the multivariate analysis $(BMI - 24.3)^2$ was the sole independent predictor of the one year ΔAF, a variable that is much easier to assess than calorie intake.

The BMI of the majority of the HD patients was within the recommended range (18 – 25). The relationship between BMI and the one year ΔAF is represented by a U-shaped curve, with the lowest point at a BMI of 24.3 kg/m². Remarkably, this level is close to the upper limit of WHO recommended range. The reason for the U-shaped relation might be that HD patients with lower BMI are relatively undernourished and can have higher endogenous production of AGEs due to the increased level of inflammation and oxidative stress, whereas the HD patients above this level have larger exogenous AGEs intake. However it is important to notes that the optimal level of BMI is close to the upper limit of the recommended range, indicating that patients with slightly increased BMI can have reduced AGEs accumulation and better outcome.

Conclusions

It appears that calorie, protein and AGE intake hardly influence the one year ΔAF in HD patients. However, BMI is a predictor of the one year ΔAF which is a strong predictor of mortality of HD patients. The BMI of HD patients of around 24 kg/m² results in the lowest one year ΔAF.

Limitations

The assessments of the dietary intake were performed at two time points with 6 months interval and were not performed at the same time as the Skin AF measurement and biochemical analysis. We assumed that no seasonal changes in the dietary intake of AGE, calories and proteins were present. Another limitation of our study is that we cannot exclude the influence of fluorophores other than AGEs on Skin AF measurements.

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Chapter 8

General discussion and future perspectives

In this study we investigated the accumulation of Advanced Glycation End-products (AGEs) in Hemodialysis (HD) patients. AGEs are an important predictor of outcome in HD patients[1,2]. In all studies AGEs were measured in the skin using AGE Reader. Additionally measured were skin AGEs directly from skin biopsies (high performance liquid chromatography and mass spectrometry), measuring pentosidine, Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL). In another study, plasma AGEs were also measured using plasma AF.

8.1. The Autofluorecence as a Method of Measurement of Advanced Glycation End-Products in Plasma and Skin

Skin Autofluorescence

Skin AGEs in all studies in this thesis were measured with the AGE Reader. The AGE Reader is a desk-top device that measures the Autofluorescence (AF) of the skin in order to quantify the level of AGEs accumulation. Initially, Skin AF results of the AGE Reader were applicable only in Caucasian subjects with a UV skin reflection > 10%. However, using a new algorithm, it was shown that results of Skin AF could be obtained to assess Skin AGEs independently of skin color for UV reflections above 6%[3]. The results of the Skin AF measurements in this study were corrected using the new algorithm. Furthermore, the measurements in this thesis were performed in Macedonia and Sweden on Caucasian subjects.

In previous validation studies using skin biopsies taken from the site of Skin AF measurements, a strong correlation was found between Skin AF and the skin contents of the fluorescent AGE pentosidine, as well as with the non-fluorescent AGEs CML and CEL[4-6]. One of these validation studies was performed in HD patients[5]. The results of **Chapter 3** provided further support for the relation between Skin AF and skin biopsy AGE levels. We performed repeated Skin AF and AGEs measurements of skin biopsies in a HD cohort and found that the rate of increase of Skin AF in HD patients had a strong correlation with the rate of increase of skin AGEs in the biopsies. The correlation between single point Skin AF measurement and the skin AGEs from biopsies was almost in line with the results of previous studies, although in our study Skin AF was measured on the forearm whereas the skin AGEs were measured in biopsies taken from the scapular region. No effect of this difference of locations was observed.

Plasma autofluorescence

In **Chapter 4** plasma AGEs were measured using AF. Plasma AF was measured at 460 nm after excitation at 370 nm. With respect to the plasma fluorophores in blood vessels it should be noted that they contribute to a much smaller extent to the measured Skin AF as

compared to fluorophores that have leaked out of the blood vessels. The large absorption of hemoglobin "hides" part of the fluorescence of the fluorophores that are within the blood vessels[7]. These findings are in line with our results from **Chapter 4**, which showed that there is no significant contribution of the changes in Plasma AF on Skin AF during a hemodialysis session.

8.2. Skin Autofluorescence Measurements Have Greater Clinical Value Than Plasma Autofluorescence Measurements

In a part of **Chapter 2** we reviewed the current knowledge about plasma and skin AGEs in Chronic Kidney Disease (CKD). Higher levels of plasma pentosidine are present in CKD patients than in matched groups of healthy controls[8]. These data are in line with those of other groups for CML in the higher CKD classes, defined by lower estimated Glomerular Filtration Rate (eGFR) in which Skin AF was independently associated by higher levels of CML in older community-dwelling adults (meaning those who are not in assisted living or nursing homes)[9]. Both diabetic and non-diabetic patients in Stage 5 of CKD, also called end-stage renal disease (ESRD), have higher levels of plasma pentosidine and CML than patients in earlier stages of CKD. Our results presented in **Chapter 4** confirm that plasma AGEs are only slightly reduced by HD[10].

The impact of AGEs-associated tissue damage is strongly dependent on the behavior of the proteins to which the AGEs are linked. The degree of AGEs accumulation and resulting damage is most evident in tissues with slow protein turnover. Several studies support that plasma or serum AGEs measurements are a poor indicator of AGEs dependent tissue damage; the clinically most relevant damage occurs in tissues with slow turnover, such as the dermis of the skin. Ueno et al reported that in ESRD patients both Skin AF and serum pentosidine correlated with carotid intima-media thickness. However, in multiple regression analysis Skin AF was independently associated with intima-media thickness whereas serum pentosidine was not[11]. Another example of this dissociation between AGEs in plasma and long-lived tissues is a study by Hartog et al in patients with heart failure, in which plasma AGEs and diastolic function were not related, whereas a strong relation existed between Skin AF and diastolic function[12]. Furthermore, serum CML did not correlate with the presence of cardiovascular disease (CVD) in a large group of HD and peritoneal dialysis patients, whereas Skin AF did[13]. Therefore it can be concluded that Skin AF, as an indicator of dermal tissue AGEs accumulation, qualifies as a better marker of tissue damage than circulating AGEs.

8.3 Can the Hemodialysis Treatment influence the Skin and Plasma Autofluorescence?

HD vintage has a positive correlation with Skin AF (**Chapter 5**, **Chapter 6**), suggesting that ESRD and the HD treatment itself contribute to AGEs formation. In **Chapter 4** we investigated the effect of mostly high flux HD on Skin AF, whereas in **Chapter 5** the effect of low flux HD was studied. These studies showed that the measured value of Skin AF was not significantly reduced neither by high flux nor by low flux HD. Nevertheless, significant changes of 14% in plasma AF by a single HD session were observed in **Chapter 4**. This 14 % reduction is significantly less than the reduction of water soluble products, such as urea. These data showed less than expected clearance of AGE products from plasma compared to products that were totally unbound. This indicates that a larger part of the AGEs present in plasma is protein bound. We can conclude that a single HD session by commonly used methods of HD is not able to influence the level of plasma AF and Skin AF.

In **Chapter 2** we reviewed the current possibilities of plasma AGEs reductions using different methods of HD. We concluded that reduction of the AGEs accumulation can be achieved by using advanced HD techniques which use membranes that have large pores such as super flux, hemodiafiltration or protein leaking membranes. The reason for the reduction of the AGEs accumulation by these techniques is their ability to remove protein-bound AGEs however the main drawback of these HD techniques is the loss of essential proteins such as albumins. Other methods that can reduce the AGEs accumulation are the use of more biocompatible membranes and ultra-pure HD fluid. The reason for the reduction of AGEs accumulation by these methods is most likely their ability to reduce the level of inflammation and immune response. Finally, use of more frequent HD regimes such as daily or home dialysis may also achieve better removal of plasma AGEs, and thus reduce AGEs accumulation. However, because of the more generalised inflammatory reaction induced by the dialysis procedure, a balance probably exists between this inflammatory response and the removal of free AGE and possibly AGE free adducts from the plasma compartment. This balance will determine the accumulation of AGE in the tissues with slow turnover such as the skin and vessel wall.

8.4 Does Hepatitis C Affect the Accumulation of Advanced Glycation End-Products in The Skin of Hemodialysis Patients?

Due to the high prevalence of Hepatitis C infection of 52% in the HD Center in Skopje we had a unique opportunity to study the influence of Hepatitis C on the AGEs accumulation in HD patients (**Chapter 5**). The Hepatitis C prevalence of HD patients in other countries is much lower[14-17].

Using single time point measurements of Skin AF (winter 2007/2008) we did not observe higher AGEs accumulation in HD patients with Hepatitis C than in patients without Hepatitis C. The studies that investigated the influence of Hepatitis C Virus (HCV) on circulatory AGEs in non-HD patients, did not show differences between healthy subjects and HCV+ patients as well[18;19]. Regarding HD patients, Nascimento et al. found higher levels of plasma pentosidine in HCV+ compared to HCV- HD patients[20]. In contrast, we did not find higher skin AGE accumulation in HCV+ compared to HCV- HD patients.

The reason for not finding elevated Skin AF in HD patients with Hepatitis C in comparison with HD patients without Hepatitis C can be found in the progression of the nature of Hepatitis C viral (HCV) infection in HD patients. Okuda K et al. compared the progress of Hepatitis C in HD patients with non-uremic controls over a period between 4 and 23 years. During the first 4 years, 25 % of the controls developed cirrhosis whereas the HD patients did not. Also, it is interesting that Okuda et al. found that all of the patients that were followed for more than 15 years had asymptomatic HCV infection[21]. These results were confirmed by our study as all HCV+ patients that were more than 9 years on HD had asymptomatic HCV infections. This suggests that the effect of the HCV infection was not severe and that HCV contributed only to a minor extent to the morbidity of the patients that were longer on HD.

For the purpose of our studies we also did repeated measurements of Skin AF in HD patients with and without Hepatitis C at approximately six months intervals, twice in winter and twice in summer. A portion of the results derived from these measurements are not presented in the previous chapters. These results showed significantly higher Skin AF values in the summer Skin AF measurements as compared to the winter Skin AF measurements: winter 2007/08 vs. summer 2008 (3.26 ± 0.81 vs. 3.48 ± 0.81: $p>0.01$), and winter 2008/09 vs. summer 2009 (3.38 ± 0.75 vs. 3.60 ± 0.82; $p>0.01$). In **Chapter 6** we found a significant increase of Skin AF in the measurements which were one year apart. The mean one year increase of Skin AF of both years was 0.17 ± 0.05. The new results showed that there are seasonal fluctuation which were defined as the additional half year increase above the expected value for a constant linear increase [AF2-(AF3-AF1)/2-AF1 or AF3-(AF4-AF2)/2-AF2] with a value of 0.19 ± 0.10. We analyzed the factors that influenced the seasonal fluctuations of Skin AF for the entire population, and after doing univariate regression analysis we found that significantly higher seasonal fluctuations of Skin AF in HD patients with Hepatitis C than in HD patients without Hepatitis C (0.31 ± 0.62 vs. -0.03 ± 0.48; $p = 0.01$). In the multivariate regression model, we found that smoking, Hepatitis C and HD vintage are independent predictors ($R^2 = 0.51$) of seasonal fluctuations of Skin AF. In figure 1 we graphically presented the impact of Hepatitis C on Skin AF in HD patients. The figure shows clearly that seasonal fluctuations are only present in patients with Hepatitis C; patients without Hepatitis C do not have seasonal fluctuations. It is worth noting that despite this seasonal fluctuation there were no significant differences between the Skin AF values of HD patients with and without Hepatitis C at any of the 4 time points or its increase per year.
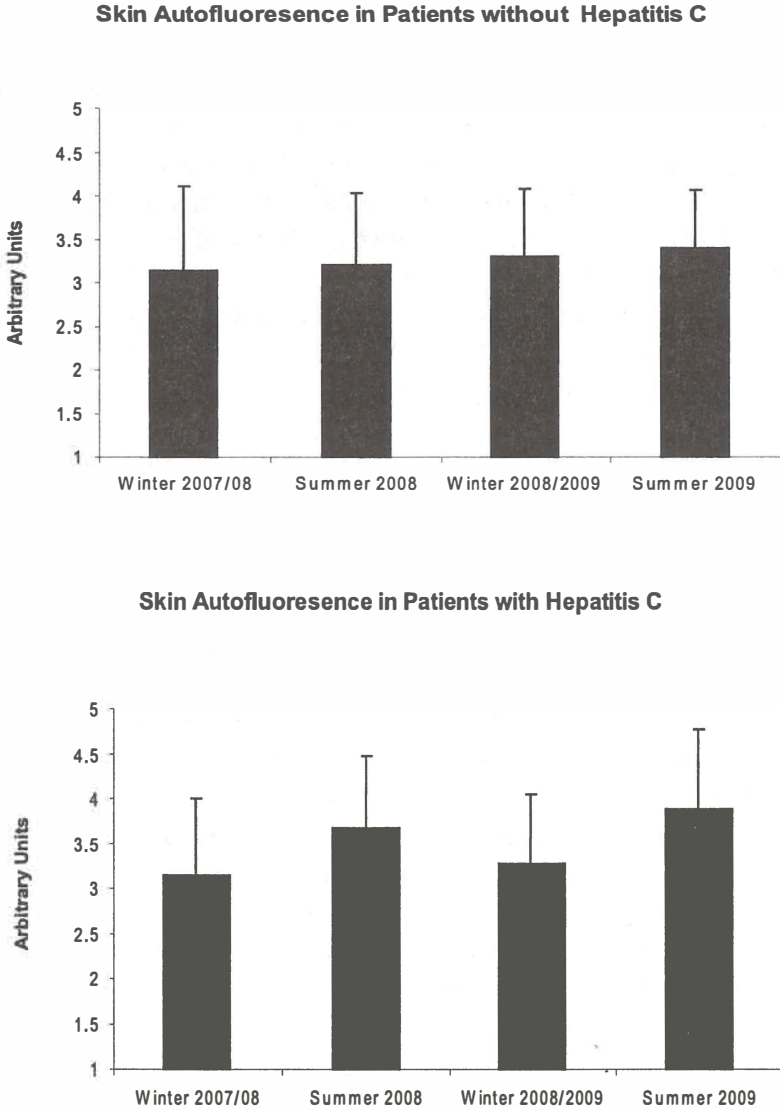
Skin Autofluoresence in Patients without Hepatitis C

Skin Autofluoresence in Patients with Hepatitis C

Figure 1. The Skin AF at the four time points at HD patients with and without Hepatitis C. In Hepatitis Virus C+ patients there are seasonal fluctuations of Skin AF, a lower than expected increase even a decrease of Skin AF in winter and higher than expected increase in summer, compared to almost the linear increase of Skin AF in Hepatitis Virus C- patients.

Because Hepatitis C is generally not very common in HD patients in Europe, we did not include the seasonal fluctuations in the paper of **Chapter 6**. Nevertheless, we investigated possible explanations for the phenomenon of seasonal fluctuation of Skin AF in HD patients with Hepatitis C. One explanation can be found in the seasonal fluctuations of bilirubin as a results of sunlight break down[22]. The sunlight breaks down bilirubin, hence higher bilirubin in winter and lower bilirubin levels in summer are expected. Bilirubin shows high absorption at wavelengths of around 450 nm[23], where the measurement of AGEs reflected fluorescent lights takes place. Thus higher bilirubin can result in reduction of the measured Skin AF value and seasonal fluctuation in the Skin AF values of HD patients with Hepatitis C.

8.5. Skin Autofluorescence as a Predictor of Mortality in Hemodialysis Patients

Meerwaldt et al were the first to show that a single point Skin AF measurement was a strong and independent predictor of overall and cardiovascular mortality in ESRD patients[5]. Jiang et al recently reported a strong predictive power of Skin AF levels in a large cohort of maintenance dialysis patients (613 peritoneal dialysis and 1775 HD)[13]. In **Chapter 6** we compared the predicating power of a single point measurement of Skin AF with repeated Skin AF measurements and found that repeated Skin AF measurements (one year increase of Skin AF) are a stronger predictor of overall and cardiovascular morality. It appears that more frequent measurements of Skin AF achieve stronger predicting power of overall and cardiovascular mortality in HD patients.

8.6. Does Nutrition influence the Accumulation of Advanced Glycation End-products in Hemodialysis Patients?

In **Chapter 3 and 7** we investigated the influence of AGEs intake on the accumulation of AGEs in HD patients, based on the observation of Uribarri et al [24], who showed for the first time that AGE intake correlates with plasma AGE levels in ESRD patients. We assessed the relationship between the rate of increase of Skin AF in time and the AGE intake in the HD population in Skopje. The one year increase of Skin AF had a borderline negative correlation with AGE and calorie intake. The AGEs in the skin biopsies did not correlate with the AGE intake in the (diabetic) HD patients, which confirms that the effect is small and does not reach significance for this small group size.

In **Chapter 7** we assessed the relationship between the one year increase of Skin AF (ΔAF) and the nutritional state in HD patients, estimated by the Body Mass Index (BMI). In the multivariate analysis we found that $(BMI - 24.3)^2$ was the sole independent predictor of

the one year increase of Skin AF. Remarkably, the level of 24.3 kg/m² is close to the upper limit of the WHO recommended range. The reason for the U-shaped relation might be that HD patients with lower BMI are relatively undernourished and can have higher endogenous production of AGEs due to the increased level of inflammation and oxidative stress, whereas the HD patients above this level may have larger AGE levels because of (exogenous) AGEs intake. From our results it can be concluded that the optimal BMI for HD patients is around 24.3 kg/m².

8.7. Future Perspectives

In Chapter 2 we presented some possible directions for future research. More frequent and repeated measurement of Skin AF in CKD patients can give insight in many factors that influence the AGEs accumulation in these patients. An obvious possible line of further research can be the influence of drugs such as sevelamer (a calcium based phosphate binding drug) in CKD, also in lower classes of CKD like class 3, on the accumulation of AGEs measured by repeated Skin AF measurement. In ESRD the influence of different dialysis methods such as super flux HD, hemodiafiltration, protein leaking HD, daily and home HD or use of ultrapure fluid on the rate of increase of Skin AF warrants further investigation.

Further questions also rose from the studies presented in this book. What is the reason for stronger mortality predicting power of Skin AF than of direct AGEs measurements? Why HCV infection has such a benign form in HD patients compared to non-HD patients? Can optimization of nutrition such as optimal BMI or AGEs intake result in a lower rate of AGEs accumulation? What are the contributions of exogenous intake and endogenous production of AGEs to the total rate of accumulation of AGEs?

Skin AF is the fastest and easiest AGEs measurement due to its optical and non-invasive nature. Skin AF also has a value outside of CKD. Of course, Skin AF measurements can also be used in other conditions that are characterized by high oxidative and glycemic stress. Skin AF can be used in the general population for assessing the future risk of cardiovascular, diabetic and renal disease.

In conclusion, with respect to the findings in this thesis, we believe that non-invasive AGEs measurement will become a valuable diagnostic tool for HD patients.

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Summary

Chronic Kidney Disease (CKD) represents a serious health and social problem. In this thesis we focused on the final stage of CKD, end-stage renal disease (ESRD). Subjects suffering from ESRD can only survive when they receive renal replacement therapy. Hemodialysis (HD), is the most frequently used method (around 70%) of renal replacement therapy. The solutes that need to be removed by HD in case of ESRD are so-called uremic toxins. Advanced Glycation End-products (AGEs) are metabolic end products that belong to the group of protein-bound uremic toxins. AGEs accumulate in tissue where they cross-link with e.g. collagen or interact with specific receptors, inducing tissue stiffening of blood vessels and skin. Current HD techniques are unfortunately only able to clear a portion of the AGEs from plasma. Skin AGE levels proved to be strong predictors of survival in HD patients. Skin AGEs can be measured by means of Skin Autoflourescence (AF). This dissertation aimed at resolving research questions, as introduced in Chapter 1, finding factors that influence the Skin and plasma AGEs and possible strategies in reducing the AGEs levels in HD patients.

In Chapter 2 we reviewed the current knowledge about plasma and skin AGEs in CKD. From the current knowledge about plasma and Skin AGEs in CKD it was concluded that Skin AF, as an indicator of dermal tissue AGEs accumulation, qualifies as a better marker of damage of various tissues than circulating AGEs. The review also presented the possibility of reducing the AGE accumulation in CKD patients using the following five methods: 1. Use of low AGE peritoneal dialysis solutions; 2. Use of advanced HD techniques; 3.Use of AGE reducing drugs; 4. Optimizing the nutrition of HD patients; 5. Renal transplantation.

The aim of Chapter 3 was to measure the accumulation of different AGEs over a period of one year in the skin of diabetic HD patients and to find the factors that influence their accumulation. The amount of the AGEs: pentosidine, carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL) in skin biopsies was measured. Skin AF measurement was used as an additional method to estimate the AGEs accumulation in the skin. Dietary records from the HD patients were obtained to assess the calorie, protein and AGEs intake. Body Mass Index (BMI), as a measure of nutritional state, was calculated. Biochemically determined skin AGEs of the diabetic HD patients did not show a significant increase in one year time. The rate of Skin AGEs accumulation was independently associated with the rate of increase of Skin AF and nutritional state.

In Chapter 4 we investigated whether changes in Skin AF appear after a single HD session and if it could be related to changes in plasma AF. The study showed that although plasma AF decreased significantly after a single HD session, this did not lead to instant differences in the cardiovascular risk marker Skin AF. Nevertheless, adequate HD remains important also from the point of view of clearance of solutes that otherwise contribute to the progression of cardiovascular morbidity.

Chapter 5 focused on the influence of Hepatitis C on AGE accumulation in HD patients. AGEs accumulation measured by means of Skin AF did not differ in HD patients with and without Hepatitis C. This might be due to the fact that hepatitis C did not cause increased oxidative stress in the studied HD population.

In Chapter 6 we assessed the one year increase of Skin AF in the total HD population as a measure of AGEs accumulation and various plasma markers, as predictors of mortality in HD patients. The mean one year increase of Skin AF of the HD patients was 0.16 ± 0.06, which was around 7-9 fold higher than the one year increase of Skin AF in healthy subjects. One year increase of Skin AF and plasma Heart-type Fatty Acid Binding Protein, used separately and in combination, proved to be strong predictors of overall and cardiovascular mortality in hemodialysis patients.

The aim of Chapter 7 was to estimate the influence of nutrition on the rate of increase of Skin AF in HD patients. Skin AF and BMI were measured four times at intervals of approximately half a year. Daily calorie, protein and AGEs intake were assessed from food recordings over period of one week. It appeared that calorie, protein and AGEs intake hardly influenced the one year increase of Skin AF in HD patients. However, BMI is a predictor of the one year increase of Skin AF which is a strong predictor of mortality of HD patients. The BMI of HD patients of around 24 kg/m2 results in the lowest one year increase of AF.

In Chapter 8, the main issues in this dissertation were discussed in a broader perspective and future research suggestions were mentioned. Such as, frequent and repeated measurement of Skin AF in CKD patients can give insight into many factors which influence the AGEs accumulation in these patients. One evident line of further research should be a study of the influence of drugs in CKD, including lower classes of CKD like class 3, on the accumulation of AGEs measured by repeated Skin AF measurement. As for ESRD the influence of different dialysis methods (such as super flux HD, hemodiafiltration, protein leaking HD, daily and home HD or use of ultrapure fluid) on the rate of increase of Skin AF warrants further investigation.

Further research questions that rose from the studies included in this book were also presented in Chapter 8. What is the reason for stronger mortality predicting power of Skin AF than of direct AGEs measurements? Why HCV infection has such a benign form in HD patients compared to non-HD patients? Can optimization of nutrition such as optimal BMI or AGEs intake result in a lower rate of AGEs accumulation? What are the contributions of exogenous intake and endogenous production of AGEs to the total rate of accumulation of AGEs?

— Samenvatting Nederlands —

Chronische nierinsufficiëntie vormt een ernstig sociaal en gezondheidsprobleem. In dit proefschrift richten wij ons op het laatste stadium van chronische nierinsufficiëntie, eindstadium nierfalen (ESNF). Patiënten die lijden aan ESNF kunnen niet overleven zonder niervervangende therapie. In dit proefschrift ligt de nadruk op hemodialyse (HD), de meest gebruikte vorm (rond 70%) van niervervangende therapie. De opgeloste stoffen die uit het bloed verwijderd moeten worden door middel van HD in geval van ESNF zijn de zogenoemde uremische toxines. Advanced Glycation End-products (AGEs) zijn metabole eindproducten die behoren tot de groep van eiwitgebonden uremische toxines. AGEs stapelen zich in de weefsels, waar zij zich binden aan bijvoorbeeld collageen of aan specifieke receptoren. Deze binding induceert verstijving van de bloedvaten en de huid. Helaas kan met de huidige dialysetechnieken alleen een deel van de AGEs uit het plasma verwijderd worden. De hoeveelheden AGEs in de huid bleken een goede indicator te zijn van de overleving van HD-patiënten. De hoeveelheden AGEs in de huid kunnen gemeten worden door middel van een autofluorescentie (AF)-meting van de huid. Dit proefschrift beoogt de in hoofdstuk 1 beschreven vragen te beantwoorden, namelijk welke factoren zijn van invloed zijn op de AF van de huid en op de hoeveelheid AGEs in het plasma, en hoe kan de hoeveelheid AGEs in HD-patiënten verminderd worden.

In hoofdstuk 2 geven we een overzicht van de huidige kennis over AGEs in bloedplasma en in de huid bij patiënten met chronische nierinsufficiëntie. Op grond van de bestaande kennis kan geconcludeerd worden dat de huid-AF, als indicator van de hoeveelheid gestapelde AGEs in de huid, een betere indicator is van weefselschade dan de hoeveelheid AGEs in de circulatie. Uit de literatuur komen de volgende vijf methoden die gebruikt zouden kunnen worden om AGE-stapeling bij ESNF-patiënten te verminderen, naar voren: 1. Het gebruik van AGE-arme spoelvloeistoffen voor peritoneaaldialyse; 2. Het gebruik van meer geavanceerde HD-technieken; 3. Het gebruik van AGE-verminderende medicatie; 4. Het optimaliseren van de voeding van HD-patiënten; 5. Niertransplantatie.

Het doel van hoofdstuk 3 was om de stapeling van verschillende AGEs in de huid van HD-patiënten met diabetes mellitus in een periode van 1 jaar te meten en factoren te vinden die de stapeling van die AGEs beïnvloeden. De hoeveelheid van de AGEs pentosidine, carboxymethyllysine (CML) en carboxyethyllysine (CEL) in huidbiopten werd gemeten. Daarnaast werd huid-AF gebruikt als een aanvullende methode om de hoeveelheid AGEs in de huid te beoordelen. Dieetdagboeken van de HD-patiënten werden gebruikt om de calorie-, eiwit- en AGEs- inname in te schatten. Tevens werd de Body Mass Index (BMI) berekend, als maat voor de voedingstoestand. De biochemisch gemeten hoeveelheden AGEs in de huid van de patiënten met diabetes mellitus lieten geen significante toename zien over een periode van een jaar tijd. De toename in huid-AF en voedingstoestand zijn onafhankelijke variabelen die van invloed zijn op de mate van stapeling van AGEs in de huid.

In hoofdstuk 4 hebben we onderzocht of na een enkele keer dialyseren veranderingen in huid-AF meetbaar zijn. Daarnaast hebben we gekeken of veranderingen in huid-AF gerelateerd zijn aan veranderingen in de plasma-AF. Uit de studie bleek dat hoewel de plasma-AF significant afnam na een enkele keer dialyseren, dit niet leidde tot acute veranderingen in de cardiovasculaire risico-indicator huid-AF. Niettemin blijft adequate HD

belangrijk, ook vanuit het oogpunt van klaring van substanties die op andere wijze bijdragen aan de progressie van cardiovasculaire morbiditeit.

In hoofdstuk 5 lag de focus op de invloed van hepatitis C op de stapeling van AGEs bij HD-patiënten. Er was geen verschil in stapeling van AGEs, gemeten middels huid-AF-metingen, tussen HD-patiënten met hepatitis C en HD-patiënten zonder hepatitis C. Wellicht veroorzaakt hepatitis C geen verhoogde oxidatieve stress bij de onderzochte populatie van HD-patiënten, waardoor deze resultaten verklaard zouden kunnen worden.

In hoofdstuk 6 onderzoeken we de toename van huid-AF in een jaar en verschillend markers in het bloedplasma bij de HD-patiëntengroep als maat voor AGE-stapeling. De AGE-stapeling en de gemeten markers zijn indicatoren van mortaliteit bij HD-patiënten. De gemiddelde toename van huid-AF in een jaar in de gehele onderzochte populatie van HD-patiënten bedroeg $0,16 \pm 0,06$. Dat is 7-9 keer zo veel als de jaarlijkse toename van huid-AF bij gezonde proefpersonen. De jaarlijkse toename van huid-AF en de hoeveelheid Heart-type Fatty Acid Binding Protein in het plasma zijn sterke voorspellers van de algemene en cardiovasculaire mortaliteit bij HD-patiënten wanneer deze voorspellers individueel gebruikt worden, maar ook wanneer zij gecombineerd toegepast worden.

Het doel van hoofdstuk 7 was een inschatting te maken van de invloed van voeding op de toename van de huid-AF bij HD-patiënten. De huid-AF en BMI werden vier keer gemeten met tussenpozen van ongeveer een half jaar. De dagelijkse calorie-, eiwit- en AGE-inname werden gemeten op basis van voedingsdagboeken die een week lang bijgehouden werden. Het bleek dat calorie-, eiwit- en AGE-inname nauwelijks van invloed waren op de jaarlijke toename van huid-AF bij HD-patiënten. De BMI daarentegen bleek een voorspeller van de jaarlijkse toename in huid-AF, en tevens een sterke voorspeller van mortaliteit bij HD-patiënten. Een BMI van rond 24 kg/m2 resulteert in de kleinste toename in huid-AF in een jaar bij HD-patiënten.

In hoofdstuk 8 worden de belangrijkste punten van dit proefschrift in een breder perspectief geplaatst en suggesties voor toekomstig onderzoek worden gegeven. Hogere absolute aantallen metingen en meer frequente metingen van huid-AF bij patiënten met chronische nierinsufficiëntie kunnen meer inzicht geven in de vele factoren die de AGE-stapeling bij deze patiënten beïnvloeden. Een logische toekomstige onderzoeksvraag kan zijn wat de invloed is van medicatie bij chronische nierinsufficiëntie op de stapeling van AGEs, ook bij lagere stadia van chronische nierinsufficiëntie zoals klasse 3. Ook vraagt de invloed van de verschillende dialysemethodes die gebruikt worden bij ESNF, zoals super-flux HD, hemodiafiltratie, protein leaking HD, dagelijkse thuisdialyse of het gebruik van ultrapure dialysevloeistof, op huid-AF om nader onderzoek. De voor dit proefschrift uitgevoerde studies brachten weer nieuwe vragen naar voren. Waarom is de voorspellende waarde van huid-AF-metingen voor mortaliteit hoger dan de voorspellende waarde van directe AGE-metingen? Waarom verloopt hepatitis C zo mild bij HD-patiënten in vergelijking tot patiënten die geen HD ondergaan? Kan voedingsoptimalisatie, zoals optimalisatie van de BMI of optimalisatie van de AGE-inname, resulteren in lagere AGE-stapeling? Wat zijn de bijdrages van exogene inname en endogene productie van AGEs op de totale hoeveelheid AGE-stapeling?

Acknowledgments

This journey is a big decision in my life. But I have faith that this must be a worthy adventure that would not only transform my thought into a broader way of thinking, but also become a precious voyage to find, understand and challenge myself.

A project like a PhD thesis of course is never the work of a single person. Many people, some in the foreground some in the background, have contributed in a way or another to this work. Here this people shall be mentioned and given my personal thanks.

Gerhard my supervisor and promoter, a very special person who welcomed me to this country has put his best efforts to challenge me. Gerhard`s patience and sense of humour made working on the project a joyful thing. His positive thinking and consistence in running the plans have been a good example for me to envision everything as a nice thing instead of seeing it as a forceful obligation. His wisdom and openness has also taught me to focus on finding the best solution out of the mistakes I made. I would like to thank him for the help he gave to me when I needed it.

Reindert, thank you for being my supervisor. You give me new insides in Skin Autofluorescence and AGEs. Even thou it took me some time to understand Reindert`s ideas, I finally found my way after creating a mountain of graphs and charts. I am grateful to Reindert for his challenge to work on this exiting subject. I am also thankful for his kindness to correct my English on my articles in the middle of his busy time. His stride for perfection made me more refined in my work and approach to science. Our cooperation gave birth to the international AGEs consortium. I would also like to thank his wife for inviting me for dinner several times.

Andries was my "resident clinician" in Groningen. I came to him for opinion and suggestions for any kind of clinical problem. The speed, precision and easiness of the communication and exchange of scientific knowledge is something that I would remember of a long time. I am very grateful for his help.

Wim introduced me to lab work. In his lab most of the blood analyses used in this thesis were performed. I would like to thank him for all the patience that he had with my two left hands. Also his input from biochemical point of view in the writing of this thesis was of great value.

Gu helped me to get the first insides into scientific work. I used his help and the old porcine experimental sample when I was doing my baby steps in the scientific world.

Lada Trajcevska, Selim Gjulsen, Pavlina Dzekova and Irena Busletic from Department of Nephrology in Skopje, Macedonia were the practical clinical side of this project. It is said that the greatest scientific lab is the patient`s bedside. I was lucky to have my "lab" in my home town of Skopje. The colleagues there helped me to collect patients` data and follow the patients` outcome. I would like especially to thank **Lada** on her hard work on the food study from this thesis.

Prof. Aleksandar Sikole was the initiator of this project. His contacts with Gerhard made it possible for me to go to Groningen and work on this thesis. During the courses of the project his inputs for clinical perspective steered this project on the right course. I

appreciate and I am thankful for his valuable criticism on my articles and support in writing the mortality study.

Prof. Bernd Stegmayer was the back bone of the AGEs Consortium. His ideas and suggestion on every chapter gave the clinical impact on the entire thesis book. His work on the chapter on High and Low flux hemodialysis and AGEs was superb. I would also like to thank him to the nice and productive time that I had in Umeå, Sweden.

Prof. Jorg Vienken made it possible for me to obtain the Fresenius Medical Care Nephrocore Scholarship. I am looking forward to our future cooperation.

Prof. Casper Schalkwijk provided me with the use of his excellent laboratories in Maastricht in which specific AGEs were measured in skin biopsy samples.

Adria Lab and **Prof. Tosko Gruev** provide me with theirs labs in Skopje where I stored and shipped my samples to The Netherlands.

Biljana Petkovska Ogjnanova is my aunt and the general practitioner in which office in Skopje I did Skin AF measurement of healthy subject. Thanks, Aunty.

I would like to thank the reading committee members **Profs. Bank, Bilo** and **Wolfenbuttel** for taking the time to evaluate my thesis.

Whether named or not, all colleagues at the department of Biomedical Engineering are acknowledged; some of you for administrative support, other for work-related help, and all of you for just being a part of the good atmosphere. With time, certain of you become more than "just" colleagues; you became friends. **Ward, Marten, Sandra, Oana, Shanti, Yun, Joana, Ed, Brandon, Otto, Jan**, **Arina, Deepak, Gerwin, Das, Agnieszka, Karsten, Adam**, **Adhi** thanks for all the fun!

Arina Buizer and **Brandon Peterson** were my paranymphs. Being away from Groningen for the final stages of the PhD project the burden of the work that they performed was much increased. Therefore I am especially grateful for their efforts.

Also I would like to thank **Vasko Kuculovski** and **Aleksandar Cakalarovski** for their help with the book and cover design.

Sandra, without you I would have quitted my PhD a thousand times. You gave me the energy to go on and you always talked me around when I was about to give up. You being a doctor helped me a lot to better explain and understand many new clinical phenomena.

Mom, Spire and Granny Vera we were far apart and I wish we could meet more often. Luckily nowadays it is quite simple to stay in touch. Your continuous support and encouragement kept me going all these years. The loss of my **Father** made us stronger and closer. **Dad** I will always miss you.

Stefan

The road of life twists and turns and no two directions are ever the same. Yet our lessons come from the journey, not the destination.

Don Williams, Jr.

List of Abbreviations

ΔAF	increase of skin autofluorescence
AF	autofluorescence
AFR	autofluorescence reader
AGE	advanced glycation end-products
ALT	alanine aminotransferase
AP	alkaline phosphates
ASA	American Society of Anesthesiologists
AST	aspartate aminotransferase
AU	arbitrary units
BMI	body mass index
CEL	$N\varepsilon$-carboxyethyl-lysine
CKD	chronic kidney disease
CML	$N\varepsilon$-carboxymethyl-lysine
CVD	cardiovascular disease
DCCT-EDIC	Diabetes Control and Complications Trial - Epidemiology of Diabetes Interventions and Complications
DM	diabetes mellitus
ELISA	enzyme-linked immunosorbent assay
ERA-EDTA	European Renal Association - European Dialysis and Transplant Association
ESRD	end-stage renal disease
FDA	Food and Drug Administration
(e)GFR	(estimated) glomerular filtration rate
γ-GST	gamma glutathione-S-transferase
HbA1C	glycated hemoglobin concentration
HbSAg	hepatitis B surface antigen
HCV	Hepatitis C Virus
HD	hemodialysis
HDF	hemodiafiltration

HDL	high-density lipoprotein
H-FABP	heart-type fatty acid binding protein
HPLC	high performance liquid chromatography
hs-CRP	high sensitive c-reactive protein
ICAM-1	inter-cellular adhesion molecule 1
KDOQI	Kidney Disease Outcomes Quality Initiative
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LED	light-emitted diode
MPO	myeloperoxydase
PD	peritoneal dialysis
RAGE	receptor of advanced glycation end-products
ROC	receiver operator characteristic
SAF	skin autofluorescence
SLC/MS	stable-isotope-dilution liquid chromatography tandem mass spectrometry
SOD	superoxide dismutase
STEMI	st-elevation myocardial infarction
UKPDS	United Kingdom Prospective Diabetes Study
USB	universal serial bus
UV	ultraviolet
vWF	von Willebrand factor
WHO	World Health Organisation