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Jasper W.L. Hartog

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‘La seule énumération de ces conséquences,
dont plusieurs peuvent être considérées comme évidentes,
serait ici trop longue’

Louis Camille Maillard (1878-1936)

Compte-rendu de l’Academie des Sciences 1912;154:66-68

Aan opa

Contents

Introduction

Advanced glycation end-products (AGEs), often referred to as glycated proteins, are molecules formed during a non-enzymetic reaction called the Maillard reaction. It was the French chemist Louis Camille Maillard who in 1912 for the first time reported on the "action des acides aminés sur les sucres" (the reaction between amino acids and sugars).[1] Although Maillard already recognized the possible importance of this reaction in disease states, it took at least 8 decades before the Maillard reaction reached awareness in clinical medicine. Over the recent years there has been an increasing interest in AGEs as can be concluded from the increased rate in publications per year (figure 1).

AGEs accumulate in the human body with age, and accumulation is accelerated in the presence of diabetes mellitus. Therefore, it is not surprising that the main focus of AGE related research for years has been the field of diabetes mellitus (figure 1). Enhanced AGE-accumulation is not restricted to patients with diabetes, but can also occur in renal failure, enhanced states of oxidative stress, and by an increased intake of AGEs. However, far less attention was directed at other fields of interest being renal disease and cardiovascular disease. The aim of the present thesis was to investigate the possible role of AGEs in renal and cardiovascular disease, in particular renal failure and heart failure.

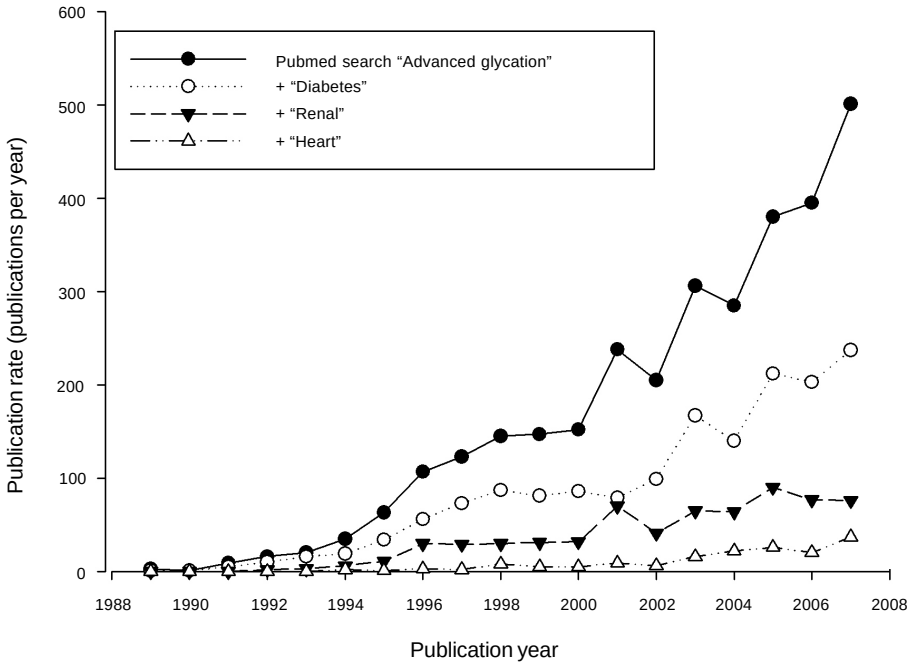

Figure 1. Publication rate for AGE related articles over recent years

Figure 1 provides the publication rate for AGE related articles over recent years. Results were obtained by introducing the search terms "advanced glycation" either alone or in combination with "diabetes", "renal" or "heart" in the PubMed database (http://www.pubmed.org).

In part one of this thesis we investigated the possible role of AGEs in heart failure. Chapter 1 provides an overview of basic AGE physiology and the pathophysiological role that AGEs may play in the development and progression of heart failure. In chapter 2 the effect of AGE-accumulation on cardiac function was studied in a dialysis population at risk for an increased AGE-accumulation. Chapter 3 focuses on a possible AGE lowering intervention by examining the effect of an AII receptor antagonist on AGE levels and cardiac function. In chapter 4 we investigated the predictive value of AGEs in chronic heart failure.

Part 2 of this thesis deals with the possible role of AGEs in the development and progression of renal failure, in particular after renal transplantation. In chapter 5 the hypothesis that AGE-accumulation can cause chronic renal transplant dysfunction is substantiated. In chapter 6 we studied the influence of renal transplantation on AGE levels. Chapter 7 focuses on the determinants of skin-autofluorescence, a measurement of tissue AGEs in renal transplant recipients. Finally, in chapter 8, we studied the predictive value of skin-autofluorescence for graft loss.

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Part I

AGEs in Cardiac Failure

Chapter 1

Advanced glycation end-products (AGEs) and heart failure: pathophysiology and clinical implications

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ABSTRACT

Advanced glycation end-products (AGEs) are molecules formed during a non-enzymatic reaction between proteins and sugar residues, called the Maillard reaction. AGEs accumulate in the human body with age, and accumulation is accelerated in the presence of diabetes mellitus. In patients with diabetes, AGE-accumulation is associated with the development of cardiac dysfunction. Enhanced AGE-accumulation is not restricted to patients with diabetes, but can also occur in renal failure, enhanced states of oxidative stress, and by an increased intake of AGEs. Several lines of evidence suggest that AGEs are related to the development and progression of heart failure in non-diabetic patients as well. Preliminary small intervention studies with AGE cross-link breakers in heart failure patients have shown promising results. In this review, the role of AGEs in the development of heart failure and the role of AGE intervention as a possible treatment for heart failure are discussed.

Introduction

Advanced glycation end-products (AGEs) were first identified in cooked food as end-products from a nonenzymatic reaction between sugars and proteins called the Maillard reaction.[1] Since the discovery that this reaction also occurs in vivo, it has been suggested that AGEs may play a role in the pathophysiology of several different diseases.[2] AGEs accumulate in the human body with age, and enhanced AGE-accumulation has been reported in patients with diabetes. Consequently, the primary interest of AGE-related research has focussed on diabetes.

In patients with diabetes, enhanced AGE-accumulation is associated with the development of diabetic sequelae and adverse outcome.[3,4] One of the diabetic complications associated with AGE-accumulation is the development of cardiac dysfunction.[5] The first manifestation is asymptomatic diastolic dysfunction, which later progresses to systolic dysfunction. In the presence of other substrates for heart failure (e.g. hypertension, coronary artery disease) this can accelerate the progression of heart failure.

Enhanced AGE-accumulation is not just restricted to patients with diabetes, but can also occur in renal failure, enhanced states of oxidative stress, and as a result of an increased intake of AGEs. Therefore, AGEs may be involved in the development of heart failure in non-diabetic patients as well. Although this has been recognised by several authors, the current literature lacks a comprehensive review on the role of AGEs in heart failure.[5-8] This review will discuss basic AGE physiology, and the pathophysiological role that AGEs may play in the development and progression of heart failure. In addition, human and animal studies of the role of AGEs in heart failure will be reviewed. Finally, the possible clinical implications of AGE intervention in heart failure will be discussed.

Basic AGE chemistry and physiology

In the first step of the Maillard reaction, a sugar adduct such as glucose, reacts with a protein amino (NH2) group, to form a Schiff-base (figure 1). This reaction occurs fast, and is reversible, depending on substrate concentrations. The Schiff-base then converts into a more stable Amadori product (e.g. HbA1c). The subsequent re-arrangement of Amadori products leads to the formation of stable and irreversible AGE compounds.[1] The final step of the Maillard reaction is driven by oxidative stress, defined as a high steady state level of reactive oxygen species (ROS). AGEs accelerate oxidation, and therefore favour their own production.[1] These pathways are especially important in diabetes. In addition to carbohydrate-driven reactions, other pathways have been identified. Important intermediates in these pathways are radicalized sugar and lipid adducts, the so-called reactive carbonyl compounds. Reactive carbonyl compounds are produced from lipids or carbohydrates reacting with ROS. These carbonyl compounds subsequently react with proteins to form AGEs.[1]

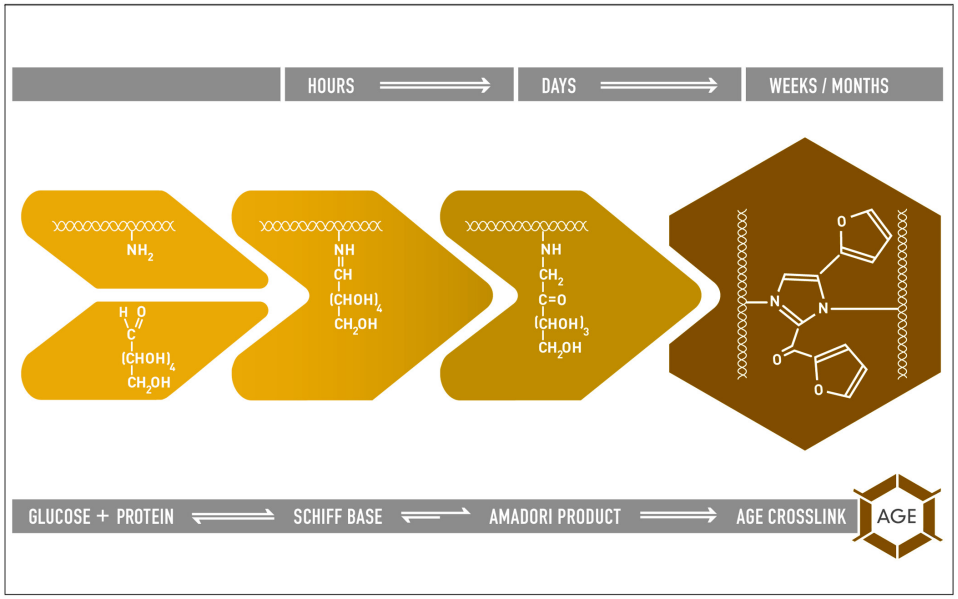

Figure 1. The Maillard reaction
Abbreviations: AGE: advanced glycation end-product.

AGEs have traditionally been detected using their fluorescence properties.[9] Recently, several mass spectrometry methods have been developed for the determination of AGE levels in both tissue and blood samples. These include gas chromatography mass spectrometry (GC–MS), and liquid chromatography mass spectrometry (LC–MS).[10] The latter is considered to be the most accurate technique available at the moment. Other methods that have been used to measure AGE levels include high performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), several immunohistochemical techniques, and techniques based upon the fluorescence properties of AGEs.[10,11] However, there are some problems associated with these techniques. HPLC although relatively accurate, is time-consuming[10] and there are difficulties associated with standardization of ELISA.[10] In addition, fluorescent techniques previously required invasive tissue sampling. Recently, these techniques have been adapted to enable their use in a clinical setting.[11] In addition to biochemical assays and fluorescent techniques, there are several immunohistochemical techniques which can be used to assess AGE levels.[12] However, these methods are not suitable for routine clinical use. Differences in the accuracy of the techniques used, should be taken into consideration when interpreting data on AGE levels.

AGE-accumulation in vivo occurs throughout the body, including the skin, neural, vascular, renal, and cardiac tissue.[13,14] Accumulation may occur within the cells or in the extra-cellular compartments. In patients with diabetes, accelerated AGE-accumulation occurs mainly as a consequence of high glucose levels.[15] Renal failure also contributes

to enhanced AGE-accumulation through decreased clearance of AGE degradation products combined with increased exposure to oxidative stress.[11,16] Cigarette smoke and heated, cooked or roasted food products are other possible sources of increased AGE-accumulation.[17,18]

PATHOPHYSIOLOGICAL EFFECTS OF AGES THAT MAY CAUSE OR ACCELERATE HEART FAILURE

Heart failure is characterised by a structural or functional cardiac disorder that results in an inability of the heart to fill with or pump out blood, combined with symptoms of dyspnea or fatigue. AGEs may contribute to the development of heart failure via two pathways. Firstly, AGEs affect the physiological properties of proteins in the extracellular matrix by creating cross-links. Secondly, AGEs cause multiple vascular and myocardial changes via the interaction with AGE receptors. AGEs may induce diastolic, systolic and vascular dysfunction through these pathways. Subsequently, these abnormalities may result in the development and progression of heart failure. A summary of these pathways is presented in figure 2.

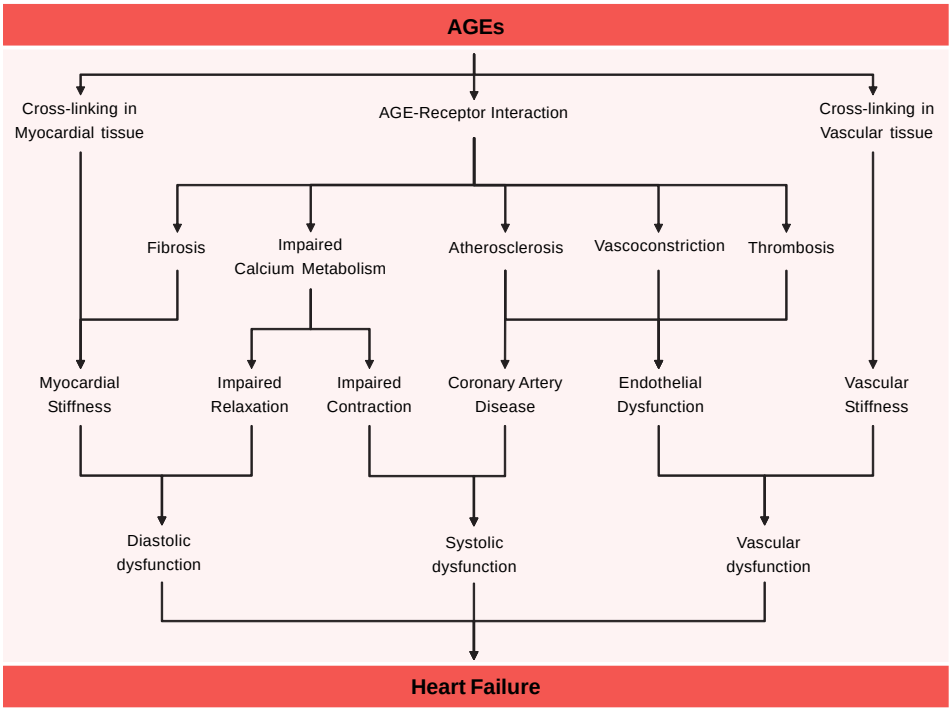

Figure 2. Summary of the pathways via which AGEs may cause heart failure
Abbreviations: AGE: advanced glycation end-product.

AGEs and diastolic dysfunction

Cross-linking of extracellular matrix proteins is essentially a physiological phenomenon. It strengthens tissues ensuring tissue integrity, without compromising flexibility. AGEs, however, can covalently bind other AGEs, and form additional cross-links between matrix proteins like collagen, laminin, and elastin.[2] Excessive cross-linking caused by AGE-accumulation undermines the flexibility of matrix proteins (figure 3a). This increased rigidity may induce diastolic dysfunction in the heart. Another pathway by which AGEs may contribute to the development of diastolic dysfunction is via the activation of AGE receptors, which have been identified on several cell-types (figure 3b).[19] The most important AGE receptor is the Receptor for AGE (RAGE). One of the receptor mediated effects of AGEs is the induction of fibrosis via the upregulation of transforming growth factor-β (TGF-β).[20] AGE-receptor activation also seems to influence calcium metabolism in cardiac myocytes. Petrova et al.[21] created transgenic mice that overexpressed human RAGE in the heart and analysed the calcium transients in cardiac myocytes in reaction to AGE exposure. RAGE over-expression was found to reduce the systolic and diastolic intra-cellular calcium concentration. Exposure to AGE caused a significant delay in calcium reuptake. As a consequence, the duration of the re-polarisation phase of the cardiac contraction may increase, subsequently causing diastolic dysfunction.

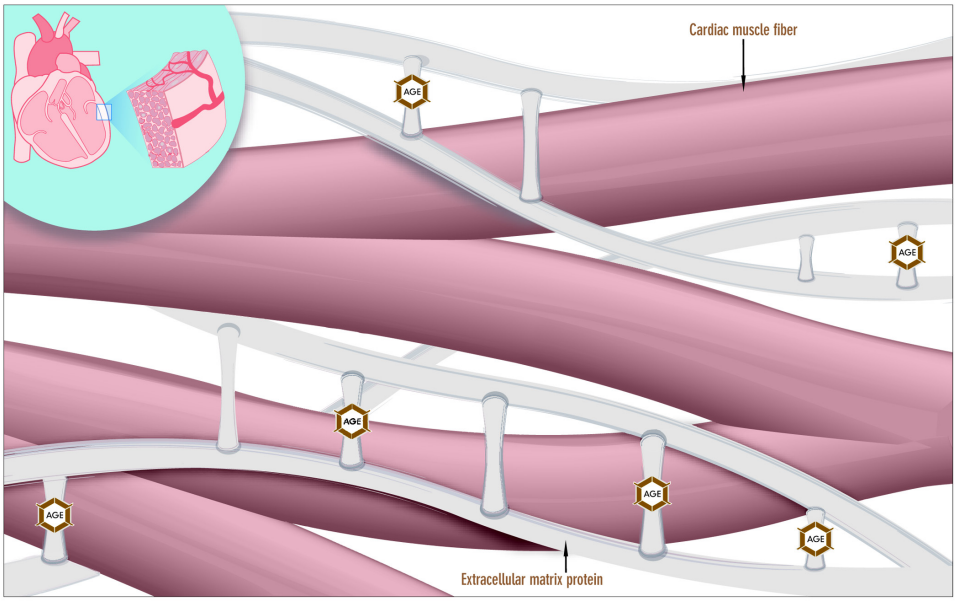

Figure 3a. AGE pathophysiology: Cross-linking

An illustration of AGE cross-linking between matrix proteins surrounding the cardiac muscle fibres. Abbreviations: AGE: advanced glycation end-product.

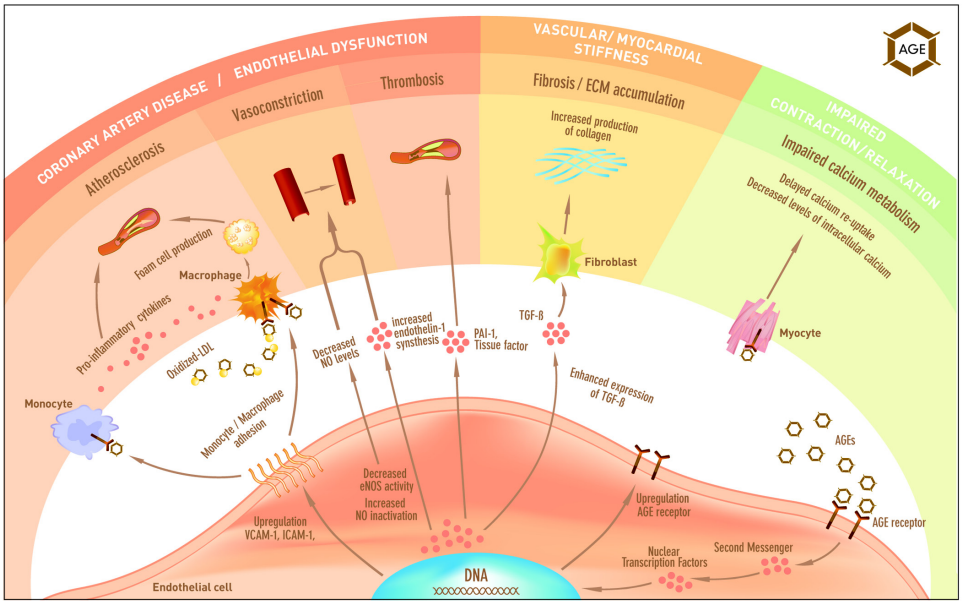

Figure 3b. AGE pathophysiology: AGE-receptor interaction

An illustration of the pathways involved in AGE-receptor interaction. AGE-receptor expression has been demonstrated on various cell-types, including endothelial cells, monocytes, macrophages, and cardiac myocytes.[19] The most important AGE receptor identified is the Receptor for AGE (RAGE). This is a multiligand receptor of the immunoglobulin superfamily. Although distinct families of ligands, among which are S100/calgranulins, amphoterin, and amyloid-β-peptide, can interact with RAGE, we will focus on the effects of AGE-ligands. Activation of RAGE stimulates second messenger pathways, among which the Ras pathway, Rac–Cdc42 pathway, Jac–Stat pathway, and the production of ROSs via the NADPH oxidase pathway.[52,53] In turn, these second messengers activate or prolong activation of nuclear transcription factors (e.g. nuclear factor-κB), that subsequently upregulate the production of endothelin-1, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, plasminogen activator inhibitor-1 (PAI-1), tissue factor, and transforming growth factor-β (TGF-β).[52,53] Activation of RAGE upregulates RAGE expression itself as well.[54] Via the pathways depicted in figure 3b, AGEs may contribute to the development of coronary artery disease, endothelial dysfunction, vascular and myocardial stiffness and impairment of cardiac contraction and relaxation. Abbreviations: AGE: advanced glycation end-product; LDL: low-density lipoprotein; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; PAI-1: plasminogen activator inhibitor-1; TGF-β: transforming growth factor-β; NO: nitric oxide; eNOS: endothelial nitric oxide synthesis; ECM: extracellular matrix.

AGEs and systolic dysfunction

AGE-accumulation may be involved in the development of systolic dysfunction by accelerating the progression of coronary artery disease. AGE-receptor interaction may induce atherosclerosis, thrombosis, and vasoconstriction (figure 3b). By negatively influencing LDL-metabolism, AGEs may further increase the risk of developing atherosclerosis and subsequent myocardial infarction.[22–24] Small soluble AGE peptides can form cross-links with low-density lipoprotein (LDL), rendering LDL particles more atherogenic and less susceptible to LDL-receptor uptake and subsequent clearance.[22] In addition, AGE modified LDL is more susceptible to macrophage uptake by AGE receptors, creating foam cells.[23,24] If AGEs play a role in the development of

atherosclerosis, one would expect AGE-lowering treatments to reduce atherosclerosis and possibly even myocardial infarction. Indeed, the use of AGE breakers and AGE-formation inhibitors in diabetic animal models reverses atherosclerosis.[25] Whether or not myocardial infarction can be prevented by AGE-lowering treatments remains to be investigated. Additionally, the reduced levels of intra-cellular calcium induced by AGEs, as discussed above, may induce systolic dysfunction due to decreased myocardial contractility.[21]

AGEs and vascular dysfunction

Endothelial dysfunction is an important predictor of adverse cardiac events, hospitalization for heart failure, and death.[26] Together with vascular compliance, endothelial dysfunction closely relates to the functional capacity of chronic heart failure patients.[27] AGEs are able to impair vascular function by influencing both endothelial function and vascular compliance (figure 3a and b). AGEs may induce endothelial dysfunction by reducing the availability of the vasodilator nitric oxide (NO).[28,29] Furthermore, AGEs can enhance the production of endothelin-1, a potent vasoconstrictor.[28,29] Vascular compliance is influenced by AGE cross-linking in a similar fashion as in myocardial tissue. In humans, levels of circulating AGEs correlate with arterial compliance.[30] Moreover, treatment with AGE breaking medication (ALT-711) improves arterial compliance in patients with vascular stiffening.[31]

Results from animal and human investigations

Diastolic heart failure

The results of studies investigating the adverse effects of AGEs on cardiac function are summarized in table 1. In rats, diastolic function measured by cardiac catheterisation has been shown to correlate with levels of carboxymethyllysine (CML), a well-known AGE.[32] Similar results were obtained in patients with type 1 diabetes mellitus.[33] Norton and colleagues[34] were the first to examine the role of AGEs in the development of diastolic dysfunction in an animal intervention study. In their experiment, the induction of diabetes in rats led to decreased compliance of the left ventricle measured with cardiac catheterisation. The authors also investigated whether captopril or the AGE-formation inhibitor aminoguanidine, could improve diastolic function. AGEs were determined by measuring myocardial collagen fluorescence. It was reported that aminoguanidine improved myocardial compliance, whereas no improvement was observed in captopril treated animals. The improvement with aminoguanidine was paralleled by a decrease in myocardial collagen fluorescence. Avendano et al.[35] confirmed these findings for aminoguanidine in a similar experiment. However, they also reported a treatment effect of ACE inhibition (using enalapril) on diastolic dysfunction and AGE-accumulation.

To date there are no published studies of the role of aminoguanidine on diastolic function in humans. Development was discontinued due to safety issues regarding the toxicity of aminoguanidine.[36] Another disadvantage of aminoguanidine is that it inhibits production of nitric oxide (NO). A well-known alternative for aminoguanidine is the

AGE-breaker alagebrium (ALT-711). Using ALT-711, Asif et al.[37] studied the effect of AGE lowering on left ventricular stiffness in aged dogs. They observed a significant reduction in age-related left ventricular stiffness measured by cardiac catheterisation after a 4-week treatment with ALT-711. These results were confirmed by Vaitkevicius et al.[38] in a similar experiment. The effect of ALT-711 on diastolic dysfunction has also been studied in humans. In the DIAMOND trial, Little et al.[39] treated 23 patients with stable diastolic heart failure with ALT-711. After 16 weeks, left ventricular mass (measured by MRI) was reduced and diastolic function (measured by tissue Doppler) had improved. Furthermore, the drug was well-tolerated and had a positive effect on patients' quality of life. The results, however, should be interpreted with caution due to the open-label study design. The PEDESTAL trial is an open-label study to investigate the effects of ALT-711 on diastolic function and left ventricular mass in patients with systolic heart failure and diastolic dysfunction. Preliminary results confirm the findings of the DIAMOND trial.[40]

Systolic heart failure

The majority of data gathered on the role of AGEs in systolic dysfunction originates from studies in diastolic dysfunction (see table 1). In studies where left ventricular systolic function was normal, AGE-lowering treatment had no effect on systolic function.[35,39,41] Interestingly, AGE-lowering therapy appears to improve systolic function in animals with systolic dysfunction. Liu et al.[42] examined the effect of ALT-711 on haemodynamic changes occurring with age in diabetic dogs which had developed marked left ventricular systolic dysfunction and aortic stiffness. ALT-711 restored left ventricular systolic function, and reduced aortic stiffness and left ventricle mass. Vaitkevicius et al.[38] previously reported a similar effect of ALT-711 in aged monkeys with a reduced left ventricular systolic function. Cheng et al.[43] investigated the effect of the novel AGE-breaker C16 and ALT-711 on diabetes induced cardiac dysfunction in rats. Four weeks of treatment with either C16 or ALT-711 both significantly improved cardiac output, reduced total peripheral resistance, and increased systemic arterial compliance. Heidland et al.[44] were the first to measure plasma AGE levels in patients with severe systolic heart failure, heart transplant recipients, and normal controls. In contrast to expectations, the authors found lower levels of plasma CML and AGE fluorescence in patients with systolic heart failure when compared to controls. Heart transplant recipients had higher levels of AGEs than controls. Possible biases, however, were hypervolaemia, lower concentration of plasma protein and decreased dietary intake of AGEs in patients with systolic heart failure. Recently, we showed that plasma levels of carboxymethyllysine (CML), a well-known AGE, correlate with NT-proBNP and NYHA functional class and predicted outcome in patients with systolic heart failure.[55] Koyama et al.[45] also evaluated the prognostic value of serum AGEs in CHF. They found that serum pentosidine levels were a significant predictor of cardiac death and re-hospitalization, independent of other known risk factors in CHF, like BNP, renal function, age, and NYHA functional class. The influence of AGE-lowering treatments in systolic dysfunction in humans are limited to the results of the PEDESTAL trial, as discussed previously, in which a trend towards an improvement in echo-cardiographic left ventricular systolic function was observed.[40]

Table 1. Animal and human studies on the role of AGEs in CHF

Reference	Study population	Study design	Results		
			AGE-accumulation	Diastolic function	Systolic function
Animal studies					
Norton et al[34]	104 rats; ST-DM; (*n=58*); C (*n=46*)	Prosp; captopril vs. AG; 4 months	AG prevented increased AGE fluor. in DM rats, effect for captopril not reported	AG prevented DD in DM rats; captopril did not	-
Avendano et al[35]	24 male dogs; alloxan induced GI (*n=16*); C (*n=8*)	Prosp.; enalapril vs. AG; 6 months	AG and enalapril prevented AGE formation	AG and enalapril prevented DD	No differences in SF were found
Asif et al[37]	20 male dogs; young C (*n=7*); old ALT-711 (*n=8*); old C (*n=5*)	Prosp.; ALT-711 vs. C; 1 month	-	ALT-711 improved DF	ALT-711 increased CO; no changes in LVEF
Vaitkevicius et al[38]	6 aged primates; ALT-711; baseline as C	Prosp.;ALT-711; 3-week treatment, 39 weeks follow-up	-	ALT-711 improved DF	ALT-711 improved SF
Liu et al[42]	12 dogs; alloxan-DM; ALT-711 (*n=5*); C (*n=7*)	Prosp.; ALT-711 vs. C; 1 month	-	ALT-711 reduced aortic stiffness; no data on DF	ALT-711 restored LVEF
Chang et al[41]	21 rats; young C (*n=7*); old AG treated (*n=7*); old C (*n=7*)	Prosp.; AG vs. C, 6 months	-	AG improved arterial function; no data on DF	No differences in CO were found
Cheng et al[43]	32 rats; ST-DM; ALT-711 (*n=8*); C16 (*n=16*); C (*n=8*)	Prosp.; ALT-711; C16 vs. C; 4 weeks	ALT-711 and C16 decreased AGE accumulation	ALT-711 and C16 improved arterial function; no data on DF	ALT-711 and C16 improved CO
Schafer et al[32]	11 Zucker diabetic rats; 11 non-obese; non DM littermates	Cross-sectional	AGEs increased in serum and cardiac tissue in DM rats.	Impaired DF in DM rats; DF correlated with cardiac AGE	No correlations between SF and AGE parameters .

Human studies					
Berg et al[33]	52 patients with type 1 DM	Cross-sectional	No controls	Serum AGE levels correlated with DF	No correlations between SF and AGE parameters
Heidland et al[44]	22 patients with advanced SHF; 30 HTX recipients; 20 C	Case-control	Lower AGEs in CHF vs. C; higher in HTX recipients	-	-
Little et al[39]	23 patients with DHF	Prosp.; Open-label; ALT-711; 16 weeks	-	ALT-711 improved DF (echo); decreased LV mass (MRI)	No differences in SF were found
Thohan et al[40]	22 patients with SHF and DHF	Prosp.; Open-label; ALT-711; 6 months	-	ALT-711 improved DF (echo); decreased LV mass (echo)	Trend to improved SF
Koyama et al[45]	141 patients with CHF and 18 C	Prosp.Cohort study; 479 days; endpoint:death/ rehospitalization	Pentosidine independently predicted endpoint	-	-
Hartog et al[55]	102 patients with SHF	Prospective	CML correlated with NYHA functional class and NT-pro-BNP and predicted outcome	-	No correlation was found between CML and LVEF

Note. Abbreviations: AG: aminoguanidine; ST: streptozotocin; DM: diabetes mellitus; C: control; Prosp: prospective; fluor: fluorescence; DF: diastolic function; SF: systolic function; DD: diastolic dysfunction; SD: systolic dysfunction; GI: glucose intolerance; LVF: left ventricular function; CO: cardiac output; LVEF: left ventricle ejection fraction; DHF: diastolic heart failure; SHF: systolic heart failure; CHF: chronic heart failure; HTX: heart transplant; LV: left ventricle; MRI: magnetic resonance imaging; CML: carboxymethyllysine; CEL: carboxyethyllysine; NYHA: New York Heart Association; NT-pro-BNP: N-terminal-pro-brain natriuretic peptide.

Discussion and clinical implications

The data presented in this review suggest that AGEs may be involved in the development of both diastolic and systolic heart failure. Evidence for a role of AGEs in diastolic heart failure is much stronger, but there is an overlap between the underlying pathophysiology of diastolic and systolic dysfunction. Diastolic dysfunction often precedes and/or accompanies systolic dysfunction. Over 90% of patients with systolic dysfunction have diastolic dysfunction as well [unpublished data]. One of the hallmarks of diastolic dysfunction is an increased left ventricle end diastolic pressure, which creates an enhanced susceptibility to develop pulmonary congestion and symptoms of dyspnea. Indeed, in patients with systolic heart failure, diastolic function and not systolic function was related to NYHA functional class and predicted exercise intolerance measured with cardiopulmonary exercise testing.[46] Therefore, we believe that the effects of AGE-accumulation on diastolic function may also have an impact on patients with systolic heart failure.

As discussed earlier, vascular function is an important determinant of morbidity and mortality in patients with heart failure. In this respect, patients with an increased afterload due to vascular dysfunction seem to be more likely to exhibit symptoms of reduced organ perfusion given a certain left ventricular ejection fraction. AGEs have shown marked influence on vascular function, and therefore, improving vascular function by AGE intervention may reduce morbidity and mortality in patients with heart failure. This, however, remains to be investigated.

The adverse effects of AGE-accumulation can be targeted in several ways. Two excellent reviews on this topic have been written by Peyroux et al.[47], and Monnier.[48] We have identified eight different AGE intervention strategies, as illustrated in figure 4. The first step in the Maillard reaction is dependent on glucose levels, thus patients with high levels of glucose are more prone to AGE-accumulation. Indeed, adequate glycaemic control in patients with diabetes mellitus can prevent increased AGE-accumulation.[3] The final step in the Maillard reaction is catalyzed by oxidative stress. Therefore, anti-oxidants (e.g. benfotiamine, carnosine, and flavonoid) are possible candidates to inhibit this step. Other drugs that prevent the latter step in the Maillard reaction are known as AGE-formation inhibitors (e.g. aminoguanidine, and pyridoxamine). ACE inhibitors and angiotensin II receptor antagonists have shown inhibitory effects on AGE formation as well.[49] AGE breaking medication (e.g. ALT-711) has the capacity to break formed AGE cross-links. Thus, this group of drugs has the potential to repair tissue damage induced by AGEs.[39,40] AGE intake can also be modulated to prevent AGE-accumulation. Both smoking and certain food products contain high levels of AGEs and AGE precursors. Smoking cessation and low-AGE diets have been shown to reduce AGE intake and thereby AGE levels in blood.[17,18] In addition, AGE-receptor interactions can be prevented in several ways. Firstly, by using a soluble form of the AGE receptor or an AGE lysozyme, AGEs and AGE precursors can be scavenged from the circulation.[47,48] Secondly, the consequences of AGEs can be prevented by blocking AGE receptors using antibodies.[47,48] Finally, intra-cellular signalling pathways upregulated by AGEs can be inhibited by AGE signal transduction inhibitors

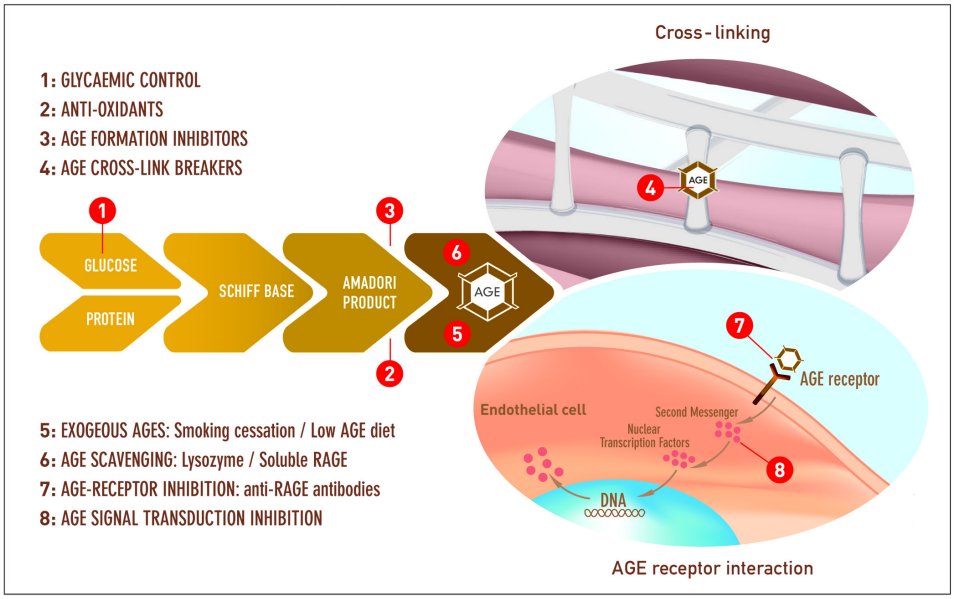

Figure 4. An illustration showing eight possible strategies for prevention of the deleterious effects of AGE-accumulation in heart failure

The Maillard reaction and subsequent processes of AGE cross-linking and AGE-receptor interaction, are shown. The numbers indicate the point of action: (1) Adequate glycaemic control in patients with diabetes mellitus reduces AGE-accumulation; (2) anti-oxidants (e.g. benfotiamine) prevent the last step in the Maillard reaction as this step is catalyzed by oxidative stress; (3) AGE-formation inhibitors (e.g. aminoguanidine, ACE inhibitors, and angiotensin II receptor antagonists) also inhibit the latter step in the Maillard reaction; (4) AGE breaking medication (e.g. ALT-711) has the capacity to break already formed AGE cross-links; (5) exogenous AGE intake can be reduced by discontinuation of smoking or the initiation of low-AGE diets; (6) AGE scavenging using a soluble form of the AGE receptor or an AGE lysozyme can prevent AGEs interacting with AGE receptors; (7) AGE-receptor inhibition or modulation can be accomplished by the use of AGE-receptor antibodies; (8) AGE signal transduction inhibition can inhibit intra-cellular pathways induced by AGE-receptor interaction. Abbreviations: AGE: advanced glycation end-product.

(e.g. incadronate disodium, cerivastatin, and cucurmin).[47,48] It should be noted that some of the drugs discussed above use more than one strategy to prevent AGE-related effects.

The cross-link breaker ALT-711 is currently being investigated for use in heart failure. ALT-711 (Alagebrium) or 4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride, is the first of a new class of thiazolium derivatives which break established AGE cross-links between proteins. By cleaving AGE cross-links, ALT-711 has the ability to restore the compliance of aged and/or diabetic vascular tissue, and myocardium. The effects of ALT-711 are not restricted to vessel tissue and myocardium, but also occur for example in skin and renal tissue. Importantly, ALT-711 does not affect the natural carbohydrate modification to proteins, intra-molecular cross-linking or peptide bonds that ensure the normal integrity of the collagen chain. Clinical experiences with ALT-711 have been favourable. Generally, ALT-711 has been reported to be safe and well-

tolerated at doses up to 210 mg, administered once or twice daily. No differences in the incidence or type of adverse events have been reported to date, in patients treated with ALT-711 vs. placebo.

Despite encouraging results, both the DIAMOND and the PEDESTAL trials have an open-label design, which is not ideal. Especially since the clinical end-points in these studies such as NYHA functional class, Minnesota Living with Heart Failure score, and exercise testing, are subjectively assessed and this may introduce bias. Double-blind, randomised, placebo-controlled trails using ALT-711 are currently ongoing.

ACE inhibitors, and angiotensin II receptor antagonists have also been shown to have inhibitory effects on AGE formation.[49] Although Norton et al.[34] did not detect a treatment effect of ACE inhibition with captopril on diastolic dysfunction in rats, Avendano et al.[35] reported an effect with enalapril in their experiments. The use of ACE inhibition in heart failure is widespread; however, the effect of ACE inhibition on AGE-accumulation in patients with heart failure remains to be investigated. It is possible that these future investigations may identify ACE inhibitors or angiotensin II receptor antagonists that lead to decreased AGE-accumulation, which may be associated with an additional beneficial effect on morbidity and mortality in heart failure patients. The other AGE intervention strategies outlined in figure 4 have not yet been investigated in heart failure.

AGE-accumulation is not restricted to specific patient groups. However, diabetic patients and patients with renal failure are especially known to have increased AGE-accumulation. Patients with these conditions also suffer from an increased prevalence of heart failure.[50] AGE-accumulation has been shown to be associated with reduced survival in patients with diabetes mellitus and patients with renal failure,[4,51] and may possibly contribute to the increased prevalence of heart failure in these conditions. Therefore, patients with diabetes mellitus or renal failure may particularly benefit from AGE intervention.

Conclusion

This review, presents the current evidence for a role of advanced glycation end-products (AGEs) in the development of heart failure. AGEs seem to be a novel and interesting new target in the treatment of chronic heart failure. In particular, the development of AGE breaking medication such as ALT-711 might prove promising for the treatment of heart failure in the future.

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Chapter 2

Skin-autofluorescence, a measure of tissue advanced glycation end-products (AGEs), is related to diastolic function in dialysis patients

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Abstract

Introduction

Diastolic dysfunction is a frequent cause of heart failure, in particular in dialysis patients. Advanced glycation end-products (AGEs) are increased in dialysis patients and are suggested to play a role in the development of diastolic dysfunction. The aim of our study was to assess whether AGE-accumulation in dialysis patients is related to the presence of diastolic dysfunction.

Methods

Data were analysed from 43 dialysis patients, aged 58±15 years, of whom 65% were male. Diastolic function was assessed using tissue velocity imaging (TVI) on echocardiography. Tissue AGE-accumulation was measured using a validated skin-autofluorescence (skin-AF) reader. Plasma N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) were measured by LC-MS/MS. Plasma pentosidine was measured by HPLC.

Results

Skin-AF correlated with mean E' (r=-0.51, $P<0.001$), E/A ratio (r=-0.39, $P=0.014$), and E/E' (r=0.38, $P=0.019$). Plasma AGEs were not significantly associated with diastolic function. Multivariable linear regression analysis revealed that 54% of the variance of average E' was explained by age ($P=0.007$), dialysis type ($P=0.016$), and skin-AF ($P=0.013$).

Conclusions

Tissue AGEs measured as skin-AF, but not plasma AGE levels were related to diastolic function in dialysis patients. Although this may support the concept that tissue AGEs explain part of the increased prevalence of diastolic dysfunction in these patients, the ambiguous relation between plasma and tissue AGEs needs further exploring.

Introduction

Systolic dysfunction is commonly recognised as the main cause of heart failure. However, approximately 50% of the patients with chronic heart failure have a preserved systolic function.[1] In most of these patients, heart failure is caused by diastolic dysfunction.[2] Recently, it became evident that the prognosis of patients with diastolic heart failure is nearly as poor as the prognosis of patients with systolic heart failure.[2-4] Despite the magnitude of this problem, little is known about the pathophysiologic background of diastolic dysfunction.

Several mechanisms underlying diastolic dysfunction have been proposed.[5-7] Diastolic dysfunction is generally associated with increased myocardial stiffness, which may be caused by modifications of collagen in the extracellular matrix. One important modification of collagen is increased cross-linking by the formation of advanced glycation end-products (AGEs). These are carbohydrate and lipid dependent modifications of protein formed by oxidative or nonoxidative reactions.[8]

AGE-accumulation occurs during life, but an increased level of AGEs has been found in patients with diabetes and renal failure. Particularly, dialysis patients are known for increased levels of AGEs, which were also independently associated with an impaired survival.[9] Interestingly, a frequent echocardiographic finding in dialysis patients is diastolic dysfunction. The prevalence of diastolic dysfunction in dialysis patients varies from 25% to 87% depending on definitions used and the patients included.[10,11]

Diastolic dysfunction predisposes to the development of heart failure and is strongly related with outcome in dialysis patients.[12] We hypothesized that increased AGE-accumulation in dialysis patients explains part of the increased prevalence of diastolic dysfunction; therefore, we analysed the relation between tissue and plasma AGEs and diastolic function in dialysis patients.

Methods

Patients and study design

In this cross-sectional study, all patients receiving dialysis treatment at the Dialysis Center Groningen ≥18 years old were eligible to participate. Both hemodialysis and peritoneal dialysis patients were included. Exclusion criteria were a myocardial infarction within the last month, significant valvular disease, pacemaker use, sustained or accepted atrial fibrillation, active endocarditis, active myocarditis, active pericarditis, acute heart failure, heart transplant, and secondary (non-idiopathic) cardiomyopathies. Non-Caucasian patients were excluded from analysis because the AGE-reader was validated only in Caucasians. Study visits as well as blood collections were performed before hemodialysis therapy. In case of peritoneal dialysis, patients' blood was withdrawn at their study visit. Using standard laboratory techniques blood was analysed for hemoglobin (Hb), HbA1c, total protein, albumin, calcium, phosphate, triglycerides, total cholesterol, and low-density lipoprotein (LDL). Furthermore, we analysed the

mean value of Kt/V per week (marker for dialysis quality based on urea clearance), which was expressed as a percentage of the Kt/V recommended by the Kidney Disease Outcomes Quality Initiative (K-DOQI) adequacy guidelines for dialysis therapy (Kt/V >3.6 for hemodialysis patients; Kt/V >2.0 for peritoneal dialysis patients). Clinical measurements were all performed on the same day as echocardiography, and included blood pressure and heart rate. In hemodialysis patients, blood pressure obtained before dialysis therapy was used for analysis. Current use of medication was extracted from medical records. History of cardiovascular disease (CVD) and family history of CVD were based on a documented or reported history of myocardial infarction, angina pectoris, cerebrovascular accident, transient ischemic attack, pulmonary embolus, venous thrombosis, or intermittent claudication in the medical history of the patient or first and second degree relatives, respectively. This study protocol complies with the Declaration of Helsinki, and was approved by the institutional review committee of the University Medical Center Groningen. All patients signed written informed consent.

AGE-accumulation measured as skin-autofluorescence

Tissue AGE-accumulation was assessed using a validated skin-autofluorescence (skin-AF) reader (AGE-Reader; patent PCT/NL99/00607; DiagnOptics BV, Groningen, The Netherlands) described previously.[9,13] In short, the AGE reader illuminates a skin surface of approximately 4 cm^2, guarded against surrounding light, with an excitation light source between 300 and 420 nm (peak excitation ~370 nm). Light from the skin is measured with a spectrometer in the 300 to 600 nm range, using 200 µm glass fiber. As a measure of skin-AF, the ratio between emission and excitation was calculated in arbitrary units (a.u.) by dividing the area under the curve between 420 and 600 nm by the area under the curve between 300 and 420 nm, and multiplying by 100. Skin-AF was measured at the volar side of the lower arm at approximately 10 to 15 cm below the elbow fold. Care was taken to perform the measurement at normal skin site (i.e., without visible vessels, scars, lichenification, or other skin abnormalities). Intraobserver variation of repeated AFR measurements on 1 day was 6%.

Plasma N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxyethyl)lysine by LC-MS/MS

Plasma N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl) lysine (CEL) were determined by stable-isotope dilution tandem mass spectrometry (LC-MS/MS) as described previously.[14] In short, 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 µmol/L nonafluoropentanoic acid as ion-pairing agent. Mass transitions of 205.1 > 384.1 and 219.1 > 384.1 for CML and CEL, respectively, and 209.1 > 388.1 and 223.1 > 388.1 for their respective internal standards were monitored in positive-ion mode. CML and CEL were separated by baseline resolution with a total analysis time of 21 minutes. Within-day and between-day coefficients of variation were <4.4% and <3.2% for CML, and <6.8% and <7.3% for CEL.

Plasma pentosidine by HPLC

Pentosidine levels were measured by high-performance liquid chromatography (HPLC) as described previously by Izuhara et al.[15] Briefly, a 50 µL solution of acid hydrolysate of plasma was injected into an high-performance liquid chromatography system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored using a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan) at an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used to obtain a standard curve. The limit of detection was 5 pmol of pentosidine per milliliter of plasma. Normal values in 4 healthy subjects averaged 0.114 ± 0.011 µmol/L, with a coefficient of variation of 5.48% ± 0.81% on 4 different days.

Echocardiography

Patients underwent 2-dimensional echocardiography, including color flow mapping 2D-guided M-mode, blood pool, and tissue Doppler echocardiography. Echocardiography was performed by experienced cardiac technicians using a General Electric VIVID 7 system with a 2.5-mHz probe. Measurements included left ventricular and atrial dimensions, the peak early (E) and late (A) diastolic filling velocities, isovolumetric relaxation time, deceleration time (slope) of the early peak filling. Furthermore, using tissue velocity imaging (TVI), early diastolic velocity (E') was measured on the lateral, septal, anterior, and inferior wall areas, and subsequently averaged. E/E' was calculated by dividing the peak early diastolic filling (E) by the average E' measured using TVI. Systolic dysfunction was defined as an estimated left ventricular ejection fraction (LVEF) ≤45%. Diastolic dysfunction was defined as an E'<8 cm/s. E' values could not be assessed in 4 patients. LVEF could not be estimated in 6 patients.

Statistical analyses

Data were analysed using SPSS version 12.01 (SPSS Inc, Chicago, Illinois). Data are reported as mean ± SD for parametric variables, as median (25%-75%, interquartile range) for nonparametric variables, and in case of nominal variables as n(%). Correlations coefficients were calculated using Pearson correlations. Linear regression analysis was used to assess the determinants of skin-AF and average E'. All variables included in table 1 were analysed by univariable linear regression. Although several echocardiographic parameters may correlate with diastolic function, by forehand, these were excluded from linear regression analysis because these would not be of interest from a pathophysiologic perspective. Only the variables that showed a trend for a relation with skin-AF or average E' ($P \leq 0.10$) were included in multivariable linear regression. Variables that did not retain significance in this multivariable analysis were subsequently removed from the model (backward selection). Next, biologically plausible but excluded variables were reintroduced in the final model to see whether their potential influences were overlooked. To test whether the model is appropriate and whether the assumptions for linear regression are met, the model was tested for overall regression, colinearity, interaction terms, and lack-of-fit with analysis of variance. Residuals were tested for normality of distribution. $P \leq 0.05$ (2-sided) was considered statistically significant.

Table 1. Patient characteristics

Characteristic	Total (n=43)
Age (years)	58 ± 15
Gender (male; n, %)	28 (65)
Dialysis type (Hemodialysis; n, %)	19 (44)
Duration of dialysis (years)	2.8 [1.3-5.3]
Kt/V per week (% of recommended Kt/V)	116 ± 30
History of renal transplantation (n, %)	6 (14)
NYHA functional class	1.3 ± 0.6
Cause of renal failure (n, %)	
Cystic renal disease	12 (28)
Hypertensive nephropathy	6 (14)
Glomerulonefritis	5 (12)
Nephrotic syndrome	3 (7)
Pyelonephritis/Reflux	3 (7)
Diabetic nephropathy	2 (5)
Other or unknown	12 (30)
Risk factors for CVD (n, %)	
History of hypertension	29 (68)
Hypercholesterolemia	19 (44)
Smoking	13 (30)
Diabetes mellitus	3 (7)
History of CVD	12 (28)
Family history of CVD	22 (51)
Physical examination	
Systolic blood pressure (mmHg)	132 ± 21
Diastolic blood pressure (mmHg)	78 ± 12
Heart rate (bpm)	77 ± 14
Body mass index (kg/m2)	25 ± 4
Laboratory assessments	
Hb (g/dl)	12.1 ± 1.6
HbA1c (%)	6.3 ± 1.1
Calcium-Phosphate product ($mmol^2/l^2$)	3.7 ± 1.0
Triglycerides (mg/dl)	168 ± 89
Total cholesterol (mg/dl)	135 ± 27
LDL cholesterol (mg/dl)	77 ± 27
Total protein (g/l)	69 ± 6
Albumin (g/l)	41 ± 4
AGE accumulation	
Skin-AF (a.u.)	3.7 ± 0.7
CML (µmol/l)	7.2 ± 2.5
CEL (µmol/l)	2.5 ± 0.6
Pentosidine (µmol/l)	1.9 ± 1.0
Medication (n, %)	
ACEi	15 (35)
β-blockers	20 (47)
Ca-antagonists	9 (21)
Diuretics	8 (19)
Erythropoietin	36 (84)
Statins	18 (42)

Abbreviations: NYHA: New York Heart Association; CVD: Cardiovascular disease; Hb: Hemoglobin; LDL: Low-density lipoprotein; ACEi: Angiotensin converting enzyme inhibitor; CML: carboxymethyllysine; CEL: carboxyethyllysine.

Results

Patient characteristics are summarised in table 1. Data were analysed from 43 patients, age 58 ± 15 years, of whom 65% were male. Mean skin-AF was 3.7 ± 0.7 a.u., which is markedly higher (P<0.001) than in a normal control group (2.0 [1.7-2.4] a.u.) previously described by our group.[16] CML (7.2 ± 2.5 vs. 2.8 ± 0.4 µmol/L; P<0.001), CEL (2.5 ± 0.6 vs. 0.8 ± 0.2 µmol/L; P<0.001), and pentosidine (1.9 ± 1.0 vs. 0.11 ± 0.01 µmol/L; P<0.001) levels were also significantly higher in patients compared with normal controls.[14,15] The plasma AGEs CML, CEL, and pentosidine showed high correlation with each other (table 2); however, they did not correlate with age and HbA1c. Dialysis quality (Kt/V) showed a strong association with plasma AGEs. Although a trend existed for CML to be related to skin-AF, no significant association existed between plasma AGEs and skin-AF.

Systolic dysfunction (LVEF ≤45%) was present in 9 (24%) patients. Diastolic dysfunction (mean E'<8 cm/s) was present in 35 (81%) patients. Skin-AF was significantly correlated with measurements of diastolic filling (table 3), including mean E' (r=-0.51; P=0.001), E/A ratio (r=-0.39; P=0.014), and E/E' (r=0.38; P=0.019). No correlations were found between LVEF, LV and atrial dimensions, and skin-AF. Plasma AGE levels were not significantly correlated with diastolic function. Plasma CEL (r=0.34; P=0.029) and pentosidine levels (r=0.31; P=0.048) did, however, correlate with LV septum diameters. Plasma CML levels showed a strong trend (r=0.30; P=0.052) for a correlation with LV septum diameters. Plasma CEL levels were additionally correlated to LV posterior wall diameters (r=0.36; P=0.02) and LA parasternal length (r=0.33; P=0.04).

Table 2. Correlations between AGE measurements, age, HbA1c and dialysis quality

	Skin-AF	CML	CEL	Pentosidine	Age	HbA1c	Kt/V
Skin-AF		r=0.27 P=0.08	r=0.02 P=0.88	r=0.24 P=0.13	r=0.46 P<0.002	r=-0.13 P=0.43	r=0.12 P=0.46
CML	r=0.27 P=0.08		r=0.55 P<0.001	r=0.85 P<0.001	r=-0.09 P=0.59	r=-0.02 P=0.93	r=-0.45 P=0.003
CEL	r=0.02 P=0.88	r=0.55 P<0.001		r=0.61 P<0.001	r=-0.08 P=0.60	r=-0.29 P=0.06	r=-0.40 P=0.009
Pentosidine	r=0.24 P=0.13	r=0.85 P<0.001	r=0.61 P<0.001		r=0.05 P=0.78	r=-0.11 P=0.49	r=-0.49 P=0.001
Age	r=0.46 P=0.002	r=-0.09 P=0.59	r=-0.08 P=0.60	r=0.05 P=0.78		r=-0.06 P=0.70	r=0.24 P=0.12
HbA1c	r=-0.13 P=0.43	r=-0.02 P=0.93	r=-0.29 P=0.06	r=-0.11 P=0.49	r=-0.06 P=0.70		r=0.29 P=0.07
Kt/V	r=0.12 P=0.46	r=-0.45 P=0.003	r=-0.40 P=0.009	r=-0.49 P=0.001	r=0.24 P=0.12	r=0.29 P=0.07	

Abbreviations: r: correlation coefficient; P: P-value; CML: carboxymethyllysine; CEL: carboxyethyllysine.

Table 3. Correlations between echocardiographic parameters and skin-AF

Parameter	Total (n=43)	Skin-AF (a.u.)	
		r	*P*-value
Diameters			
LV Septum (mm)	10 ± 2	0.11	0.49
LV Posterior wall (mm)	10 ± 2	0.13	0.43
LVEDD (mm)	46 ± 6	-0.14	0.37
LVESD (mm)	29 ± 6	-0.13	0.41
LA parasternal (mm)	38 ± 5	-0.03	0.86
LA length (mm)	56 ± 7	-0.13	0.41
LA cross (mm)	40 ± 6	-0.20	0.20
Systolic function			
LVEF (%)	53 ± 8	0.09	0.58
Diastolic function			
E (m/s)	0.74 ± 0.30	0.06	0.73
A (m/s)	0.79 ± 0.31	0.33	0.037
E/A ratio	0.98 ± 0.35	-0.39	0.014
Dct (ms)	248 ± 78	0.29	0.065
IVRT (ms)	90 ± 23	-0.05	0.78
Lateral E' (cm/s)	7.4 ± 3.1	-0.49	0.001
Septal E' (cm/s)	5.3 ± 2.0	-0.46	0.003
Anterior E' (cm/s)	6.7 ± 2.8	-0.43	0.006
Inferior E' (cm/s)	6.0 ± 2.6	-0.50	0.002
Average E' (cm/s)	6.5 ± 2.3	-0.51	0.001
E/E' (cm/s)	12.0 ± 4.6	0.38	0.019

Abbreviations: r: correlation coefficient; LV: Left ventricular; LVEDD: Left ventricular end-diastolic diameter; LVESD; Left ventricular end-systolic diameter; LA: Left atrial; LVEF: Left ventricular ejection fraction; Dct: Deceleration time; IVRT: Isovolumetric relaxation time.

Skin-AF showed a strong association with diastolic function; therefore, we performed additional analyses to determine factors that are associated with an increased skin-AF. Skin-AF was univariably associated with age, total protein, and albumin (table 4). Additionally, skin-AF showed a trend ($P \leq 0.10$) for an association with diastolic blood pressure and CML levels. Multivariable linear regression analysis revealed that 37% of the variance of skin-AF was determined by age and CML levels. Next, several biologically plausible variables were reintroduced in this model to see whether any possible relations were overlooked. Reintroducing gender, dialysis type, duration of dialysis, Kt/V, the presence of diabetes, smoking, history of hypertension, diastolic blood pressure, systolic blood pressure, HbA1c, total protein, albumin, CEL, pentosidine, statin use, and the use of angiotensin-converting enzyme inhibition showed that gender (r=0.34, P=0.011), albumin levels (r=0.28, P=0.045), and diastolic blood pressure (r=-0.27, P=0.045) all individually determined an extra part of the variance of skin-AF levels. Further multivariable linear regression analysis led to the final model in which 56% of the variance of skin-AF was determined by age, gender, diastolic blood pressure, and CML levels.

Table 4. Determinants of skin-AF and diastolic function in linear regression analysis

Characteristic	Skin-AF				Diastolic function			
	Univariable		Multivariable r^2=0.56		Univariable		Multivariable r^2=0.54	
	β	*P*	β	*P*	β	*P*	β	*P*
Age (years)	0.46	0.002	0.57	<0.001	-0.61	<0.001	-0.41	0.007
Gender (male)	0.25	0.11	0.36	0.006	-0.11	0.50		
AGE accumulation								
Skin-AF (a.u.)*	-	-			-0.59	<0.001	-0.41	0.13
CML (µmol/l)	0.27	0.08	0.35	0.006	-0.22	0.19		
CEL (µmol/l)	0.02	0.88			0.05	0.78		
Pentosidine (µmol/l)	0.24	0.13			-0.20	0.25		
Dialysis Type (Peritoneal)	-0.05	0.77			-0.23	0.17	-0.29	0.016
Duration of dialysis (years)	0.17	0.28			0.23	0.15		
Kt/V per week (%)	0.12	0.46			-0.18	0.27		
History of renal transplantation	-0.01	0.94			0.34	0.033		
NYHA functional class	-0.04	0.80			0.04	0.81		
Cause of renal failure								
Cystic renal disease	-0.21	0.17			0.07	0.69		
Hypertensive nephropathy	0.18	0.26			-0.23	0.16		
Glomerulonefritis	-0.13	0.43			0.27	0.095		
Nephrotic syndrome	0.01	0.95			-0.04	0.81		
Pyelonephritis/Reflux	-0.02	0.89			0.05	0.79		
Diabetic nephropathy	0.01	0.93			-0.15	0.37		
Other or unknown	0.16	0.29			-0.03	0.87		
Risk factors for CVD (n, %)								
History of hypertension	0.23	0.14			-0.35	0.031		
Hypercholesterolemia	-0.01	0.94			0.35	0.28		
Smoking	0.09	0.55			0.28	0.082		
Diabetes mellitus	0.15	0.35			-0.17	0.31		
History of CVD	0.12	0.45			-0.27	0.097		
Family history of CVD	0.01	0.96			-0.22	0.18		
Physical examination								
Systolic blood pressure (mmHg)	-0.06	0.70			-0.15	0.40		
Diastolic blood pressure (mmHg)	-0.32	0.054	-0.34	.008	-0.12	0.50		
Heart rate (bpm)	0.00	0.99			0.05	0.78		
Body mass index (kg/m2)	0.13	0.44			-0.30	0.077		
Laboratory assessments								
Hb (g/dl)	0.20	0.20			-0.18	0.26		
HbA1c (%)	-0.13	0.43			-0.08	0.64		
Calcium-Phosphate product	-0.08	0.62			-0.08	0.65		
Triglycerides (mg/dl)	0.20	0.20			-0.16	0.32		
Total cholesterol (mg/dl)	0.13	0.40			-0.10	0.56		
LDL cholesterol (mg/dl)	0.06	0.69			0.05	0.77		
Total protein (g/l)	0.42	0.006			-0.26	0.11		
Albumin (g/l)	0.40	0.008			-0.30	0.069		
Medication (n, %)								
ACEi	-0.20	0.20			0.17	0.30		
β-blockers	0.04	0.78			-0.05	0.77		
Ca-antagonists	0.08	0.63			-0.11	0.49		
Diuretics	-0.23	0.14			-0.06	0.73		
Erythropoietin	-0.20	0.19			0.23	0.16		
Statins	-0.01	0.97			-0.30	0.069		

Skin-AF and average E' were used as dependent variable. Only parameters that obtained at least a P-value ≤0.10 in univariable linear regression analysis were included in multivariable regression analysis. AF, autofluorence; a.u., arbitrary units; CML, carboxymethyllysine; CEL, carboxyethyllysine; NYHA, New York Heart Association; CVD, cardiovascular disease; Hb, hemoglobin; LDL, low-density lipoprotein; ACE, angiotensin-converting enzyme.
**Skin-AF (a.u.) was inversed and multiplied by -1.*

Univariable linear regression analysis revealed that diastolic function (mean E') was associated with age and history of renal transplantation, hypertension, hypercholesterolemia, and skin-AF (table 4). Furthermore, a trend ($P \leq 0.10$) for an association existed for glomerulonephritis as cause of renal failure, smoking, history of CVD, body mass index, albumin, and use of statins. Multivariable linear regression analysis showed that 45% of the variance of mean E' was determined by age and skin-AF. Reintroducing gender, dialysis type, duration of dialysis, Kt/V, the presence of diabetes, history of hypertension, HbA1c, systolic blood pressure, diastolic blood pressure, calcium-phosphate products, CML, CEL, pentosidine, and the use of angiotensin-converting enzyme inhibition showed that diastolic blood pressure (r=-0.27, P=0.036) and dialysis type (r=-0.29, P=0.016) both individually determined an extra part of the variance of diastolic function. Further multivariable linear regression analysis led to the final model in which 54% of the variance of diastolic function was determined by age, skin-AF, and dialysis type. However, diastolic blood pressure did show a non-significant contribution, thereby creating an alternative model explaining 61% of the variance of diastolic function by age (β=-0.39, P=0.009), skin-AF (β=-0.47, P=0.003), dialysis type (β=-0.26, P=0.034), and diastolic blood pressure (β=-0.22, P=0.07).

Discussion

The main result of our study is that AGE-accumulation, measured as skin-AF, is independently associated with diastolic function in dialysis patients. Age, dialysis type, and skin-AF together explain 54% of the variance of diastolic function. Plasma AGE levels were not associated with diastolic function in our study. To our knowledge, this is the first study that demonstrates the relation between diastolic function and AGE-accumulation in dialysis patients.

Diastolic dysfunction is a frequent finding in dialysis patients and is associated with an impaired survival.[10-12] We used TVI as a diagnostic tool to assess diastolic function. TVI is a noninvasive tissue Doppler echocardiographic technique that measures the myocardial tissue velocities of the mitral valve annulus during diastole. Although TVI measurement is less affected by changes in preload in opposition to conventional Doppler echocardiography, measurements are still preload-dependent. To prevent preload- dependent differences, all echocardiography measurements in hemodialysis patients were performed before dialysis therapy.

Diastolic function is strongly related to age in the general population. Although we did not use an age-dependent cutoff point to diagnose diastolic dysfunction, our results were adjusted for age. Hypertension is another well-known risk factor for diastolic dysfunction. However, we did not find a significant association between diastolic function and blood pressure, although we did find a modest association between diastolic function and a history of hypertension. Furthermore, we found that diastolic blood pressure, although insignificant, can explain an additional 7% of the variance of diastolic function in multivariable analysis.

Skin-AF was used as a measure for tissue AGE-accumulation. We previously demonstrated that skin-AF shows a strong correlation with collagen-linked fluorescence

(r=0.71, $P<0.001$), pentosidine levels (r=0.75, $P<0.001$), and levels of the nonfluorescent AGEs CML (r=0.45, $P=0.01$) and CEL (r=0.45, $P=0.01$) measured in tissue biopsies obtained from dialysis patients.[9] Furthermore, skin-AF levels were independently associated with an impaired prognosis of dialysis patients.[9]

In contrast with tissue AGE-accumulation, plasma AGEs levels were not associated with diastolic function in the present study. Both the LC-MS/MS method and the high performance liquid chromatography method used in our study to assess plasma AGEs are currently considered as the most accurate methods available. In our opinion, our findings may suggest that plasma AGE levels do not adequately reflect tissue AGE-accumulation. Indeed, CML explains only a part of measured skin-AF in our study, whereas pentosidine and CEL have not shown an independent relationship with skin-AF. Plasma AGE levels may be further influenced by dialysis modalities and absorption from food and smoking. Indeed, we showed that plasma AGEs in these dialysis patients were strongly associated with dialysis quality, but not related to traditional determinants of AGE formation such as age and HbA1c. However, we cannot exclude the possibility that a power issue may explain the lack of correlation found between plasma AGEs and diastolic function. Additionally, it would seem reasonable to assume that tissue AGEs are more closely related to diastolic function, because they are intrinsically linked with the actual pathophysiologic effects of AGEs (i.e., protein cross-linking).

Although our data limit us from drawing conclusions about causality, our results are in line with the hypothesis that AGEs play a pathophysiologic role in the development of diastolic dysfunction. AGEs may contribute to the development of diastolic dysfunction either via cross-linking of matrix molecules or via the interaction with AGE receptors. Cross-linking of extracellular matrix proteins is essentially a physiologic phenomenon. AGEs, however, can covalently bind other AGEs, and form additional cross-links between matrix proteins such as collagen, laminin, and elastin.[8] Excessive cross-linking caused by AGE-accumulation undermines the flexibility of matrix proteins. This increased rigidity may induce diastolic dysfunction in the heart. One of the receptor-mediated effects of AGEs is the induction of fibrosis via the upregulation of transforming growth factor-β.[17] AGE receptor activation also influences calcium metabolism in cardiac myocytes, causing a significant delay in calcium reuptake and consequent increase in the duration of the repolarization phase.[18]

The role of AGEs in the development of diastolic dysfunction has been investigated extensively in animals.[19-23] The effect of various AGE-lowering strategies used in these studies confirms an active role for AGEs in the development of diastolic dysfunction. Only a limited number of human studies have been published, of which none are in dialysis patients.[24-26] Berg et al[24] analysed serum AGE levels and left ventricular diastolic function in 52 patients with type 1 diabetes. The authors found a positive correlation between serum AGEs and isovolumetric relaxation time and left ventricular end-diastolic diameter. No correlation was found between AGEs and other echocardiographic parameters for diastolic function. It should be noted, however, that tissue velocity or Doppler imaging was not used to determine diastolic function in the latter study.

Recently, we showed that plasma levels of CML correlate with NT-proBNP and NYHA functional class and predicted outcome in patients with chronic heart failure.[27] The most convincing evidence for a role of AGEs in the development of cardiac

dysfunction originates from 2 trials with the AGE cross-link breaker alagebrium (ALT-711) in patients with chronic heart failure. In the DIAMOND [Distensibility Improvement and Remodeling in Diastolic Heart Failure] trial, Little et al[25] treated 23 patients with stable diastolic heart failure open-label with ALT-711. After 16 weeks, left ventricular mass (measured by magnetic resonance imaging) was reduced and diastolic function (measured by tissue Doppler) had improved. Furthermore, the drug was well tolerated and had a positive effect on patients' quality of life. The PEDESTAL [Patients with Impaired Ejection Fraction and Diastolic Dysfunction: Efficacy and Safety Trial of Alagebrium] trial is an open-label study investigating the effects of ALT-711 on diastolic function and LV mass in 20 patients with systolic heart failure and diastolic dysfunction. Preliminary results confirm the findings of the DIAMOND trial.[26] Although the results of both trials should be interpreted with caution because of the open-label design, further exploration of the effects of AGE-lowering therapies is warranted because no therapy has as yet shown effectiveness in the treatment of diastolic heart failure. A prospective randomised, double-blind, placebo-controlled trial on the effects of alagebrium on exercise tolerance and diastolic function in 100 chronic heart failure patients is ongoing (BENEFICIAL trial; www.clinicaltrials.gov; NCT00516646).

Conclusion

Tissue AGEs measured as skin-AF, but not plasma AGE levels, were related to diastolic dysfunction in dialysis patients. Although this may support the concept that tissue AGEs explain part of the increased prevalence of diastolic dysfunction in these patients, the ambiguous relation between plasma and tissue AGEs needs further exploring. Moreover, the concept that AGEs are related to the development of diastolic dysfunction needs to be established in interventional studies using AGE lowering therapies.

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Chapter 3

Skin-autofluorescence, a measure of tissue advanced glycation end-products, determines the effect of anti-hypertensive treatment on diastolic function in patients with hypertension and diastolic dysfunction

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Abstract

Introduction

Advanced glycation end-products (AGEs) have been associated with diastolic dysfunction. Angiotensin II type 1 receptor blockers (ARBs) may lower in vivo AGE formation. We investigated the relation between AGEs and diastolic function and the response to blood pressure treatment in patients with hypertension and diastolic dysfunction.

Methods

Data were analysed from 97 patients who were randomly assigned to 6 months open-label treatment with either eprosartan on top of other anti-hypertensive drugs (n=47) or other anti-hypertensive drugs alone (n=50). Tissue AGE-accumulation was measured using a validated skin-autofluorescence (skin-AF) reader (n=26). Plasma N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), and pentosidine were measured by LC-MS/MS and HPLC. Diastolic function was assessed using echocardiography.

Results

Mean age of the patients was 65±10 years, 36% was male, and mean tissue E' was 6.7±2.3 cm/s. Blood pressure was reduced from 157/91 to 145/84 mmHg (P<0.001) in the eprosartan group and from 158/91 to 141/83 mmHg (P<0.001) in the control group. No effect of eprosartan was found on skin-AF, CML, CEL, and pentosidine. In patients with baseline skin-AF < median, E/A ratio (from 0.84 [0.69-0.92] to 0.92 [0.85-1.04], P=0.04) and the mean peak early diastolic filling velocity (E') improved (from 5.9±1.4 to 7.1±1.3 cm/s, P=0.001). In contrast, in patients with skin-AF levels > median, E/A ratio (P=0.84) and mean E' (P=0.30) remained unchanged.

Conclusions

Eprosartan did not decrease tissue and plasma levels of AGEs in patients with hypertension and diastolic dysfunction. However, patients with lower skin-AF at baseline showed a larger improvement in diastolic function in response to anti-hypertensive treatment compared to patients with higher skin-AF.

Introduction

Although hypertension is generally asymptomatic, it is related to a higher risk for development of heart failure, particularly diastolic heart failure. Several mechanisms underlying diastolic heart failure have been proposed.[1-4] Diastolic heart failure is generally associated with increased myocardial stiffness, which may be caused by modifications of collagen in the extracellular matrix. One important modification of collagen is increased cross-linking by the formation of advanced glycation end products (AGEs). These are carbohydrate and lipid dependent modifications of protein, formed by oxidative and non-oxidative reactions.[5]

Recently, we demonstrated that skin-AF, a measure of tissue AGEs is strongly related to diastolic function in dialysis patients.[6] It remains unknown whether AGEs are related to diastolic function in patients with hypertension and signs of diastolic dysfunction. In vitro and in vivo studies have shown that angiotensin II type 1 receptor blockers (ARBs) can reduce AGE formation.[7-9] ARBs prevent the production of reactive carbonyl and dicarbonyl compounds (RCOs), which are critical precursors of AGEs.[7-9] However, conflicting clinical data on the effects of ARBs on AGE-accumulation have been presented.[8-11]

Therefore, the first aim of this study was to evaluate the effects of the ARB eprosartan versus control anti-hypertensive treatment on both serum and tissue AGEs in a randomised clinical study in patients with hypertension and diastolic dysfunction. Second, we aimed to establish the influence of baseline tissue and serum AGEs on changes in diastolic function in response to eprosartan and control anti-hypertensive treatment.

Methods

Patients and study design

For the present analysis, we studied 97 patients, participating in a prospective randomised open-label trial with blinded end-point (PROBE design) that aimed to establish the effects of the angiotensin II-AT1 receptor blocker (ARB) eprosartan in hypertensive patients with signs of diastolic dysfunction. Patients older that 18 years, with hypertension (blood pressure repeatedly ≥140/90 mmHg), who were not yet treated with an angiotensin II-AT1 receptor blocker, were screened for signs of diastolic dysfunction on echocardiography. Patients were eligible to participate if left ventricular ejection fraction (LVEF) was ≥50%, and E/A was <1 in combination with either a deceleration time (Dct) >280 ms or an isovolumetric relaxation time (IVRT) >105 ms. Exclusion criteria were recent myocardial infarction (<6 weeks), unstable angina pectoris, severe valvular disease, acute heart failure, atrial fibrillation, pacemaker, history of drug-sensitivity or allergy for eprosartan, pregnancy or lactation, clinical significant liver or renal disease, infection, and previous poor quality echocardiogram. Five patients were permitted study entry, although they were already using an ARB. All these patients were using ARBs for a short period (<1 months) before entry and

were therefore provided with a waiver. Patients were randomised between eprosartan 600 mg once daily (400 mg 2 weeks loading dose) on top of other anti-hypertensive drugs (n=47) or other anti-hypertensive drugs alone (n=50) for a period of 6 months. Other anti-hypertensive drugs that were allowed included ACE-inhibitors, β-blockers, diuretics, and calcium antagonists. Baseline and follow-up measurements included echocardiography, skin-AF, circulating AGEs, and basic laboratory values. Estimated GFR (eGFR) was calculated using the sMDRD formula as described by Smilde et al.[12] The severity of heart failure was classified in accordance with the NYHA functional class. This study protocol was approved by the institutional review committee and all patients signed written informed consent.

AGE-accumulation measured as skin-autofluorescence

Tissue AGE-accumulation was assessed using a validated skin-AF reader as described previously.[13,14] In short, the AGE-reader illuminates a skin surface of approximately 4 cm^2, guarded against surrounding light, with an excitation light source between 300-420 nm (peak excitation ~ 370 nm). Light from the skin is measured with a spectrometer in the 300-600 nm range, using 200 μm glass fiber. As a measure of skin-AF the ratio between emission and excitation was calculated in arbitrary units (a.u.) by dividing the area under the curve between 420-600 nm by the area under the curve between 300-420 nm, and multiplying by 100. Skin-AF was measured at the volar side of the lower arm at approximately 10-15 cm below the elbow fold. Care was taken to perform the measurement at normal skin site, i.e. without visible vessels, scars, lichenification, or other skin abnormalities. Intraobserver variation of repeated AGE-reader measurements on one day was 6%. Skin-AF data was obtained in 26 patients. (AGE-reader; patent PCT/NL99/00607; DiagnOptics BV, Groningen, The Netherlands)

Plasma N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxyethyl)lysine by LC-MS/MS

Plasma N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl) lysine (CEL) were determined by stable-isotope dilution tandem mass spectrometry (LC-MS/MS) as described previously.[15] In short, 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 μmol/L nonafluoropentanoic acid as ion-pairing agent. Mass transitions of 205.1 -> 384.1 and 219.1 -> 384.1 for CML and CEL, respectively, and 209.1 -> 388.1 and 223.1 -> 388.1 for their respective internal standards were monitored in positive-ion mode. CML and CEL were separated by baseline resolution with a total analysis time of 21 min. Within-day and between-day coefficients of variation were <4.4% and <3.2% for CML, and <6.8% and <7.3% for CEL.

Plasma pentosidine by HPLC

Pentosidine levels were measured by high performance liquid chromatography (HPLC) as described previously by Izuhara et al.[16] Briefly, a 50-μL solution of acid hydrolysate of plasma was injected into an HPLC system and separated on a C18 reverse-phase

column (Waters, Tokyo, Japan). The effluent was monitored using a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan) at an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used to obtain a standard curve. The limit of detection was 5 pmol of pentosidine per milliliter of plasma. Normal values in 4 healthy subjects averaged 0.114 ± 0.011 μmol/L, with a coefficient of variation of 5.48% ± 0.81% on 4 different days.

Echocardiography

Patients underwent 2-dimensional echocardiography, including color flow mapping 2D-guided M-mode, blood pool and tissue Doppler echocardiography. Echocardiography was performed by experienced cardiac technicians using a General Electric VIVID 7 system with a 2.5 mHz probe. Technicians were blinded for the allocated treatment. Measurements included left ventricular and atrial dimensions, the peak early (E) and late (A) diastolic filling velocities, isovolumetric relaxation time (IVRT), deceleration time (slope) of the early peak filling (DCT). Furthermore, using tissue velocity imaging (TVI), early diastolic velocity (E') was measured on the lateral, septal, anterior, and inferior wall areas, and subsequently averaged (mean E'). E/E' was calculated by dividing the peak early diastolic filling (E) by the average E' measured using TVI.

Statistical analyses

Data were analysed using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean±SD or as median [25-75% interquartile range], where applicable. Nominal variables are expressed as n (%). Baseline characteristics were analysed for difference over treatment group by using student's t test or Mann-Whitney U test where applicable for continuous variables and by chi-square using nominal variables. The effects of eprosartan on AGEs, diastolic function, and blood pressure were evaluated using student's t test or Mann-Whitney U test where applicable. Analyses were done based upon the intention to treat principle. The overall differences between baseline and follow-up values for blood pressure, AGEs, and diastolic function were analysed using paired t test as well as Wilcoxon signed ranks test as appropriate. Multivariable linear regression analysis was used to correct our results for possible confounders. The correlation between AGEs and diastolic function was assessed using Pearson and Spearman's rho correlation where applicable. A *P*-value $P \leq 0.05$ (two-sided) was considered statistically significant.

Results

Baseline characteristics are depicted in table 1. Data were analysed from 97 patients (35 male) aged 65±10 years who were randomly assigned to 6 months open-label treatment with either eprosartan and other anti-hypertensive drugs (n=47) or other anti-hypertensive drugs alone (n=50). The majority of patients were classified as NYHA functional class I (69%), whereas 29 % were classified as NYHA functional class II and 2% as NYHA functional class III. Only few patients had diabetes mellitus (11%).

Table 1. Baseline characteristics

Parameter	Total (n=97)
Age (years)	65 ± 10
Gender (male; n, %)	35 (36)
NYHA functional class	1.3 ± 0.5
Diabetes mellitus (n, %)	11 (11)
Physical examination	
Systolic blood pressure (mmHg)	158 ± 17
Diastolic blood pressure (mmHg)	91 ± 10
Heart rate (bpm)	69 ± 12
Body mass index (kg/m2)	29 ± 5
AGE accumulation	
Skin-AF (a.u.)	2.4 ± 0.4
CML (µmol/l)	1.5 ± 0.3
CEL (µmol/l)	1.5 ± 0.4
Pentosidine (µmol/l)	0.16 ± 0.09
Laboratory assessments	
Hemoglobin (g/dl)	14.3 ± 1.1
Creatinine (mmol/l)	82 ± 21
eGFR with MDRD (ml/min/1.73m^2)	76 ± 16
Echocardiography	
LVEF (%)	60 [55-60]
E (m/s)	0.69 ± 0.15
A (m/s)	0.83 ± 0.23
E/A ratio	0.82 [0.7-0.9]
Dct (ms)	243 ± 55
IVRT (ms)	110 [90-140]
Lateral E' (cm/s)	7.5 ± 2.9
Septal E' (cm/s)	6.2 ± 2.4
Anterior E' (cm/s)	6.2 ± 2.4
Inferior E' (cm/s)	6.5 ± 2.6
Average E' (cm/s)	6.7 ± 2.3
E/E' (cm/s)	11.3 ± 4.0
Medication (n, %)	
ACE-inhibitors	43 (44)
AII receptor blockers	5 (5)
β-blockers	35 (36)
Ca-antagonists	18 (19)
Diuretics	30 (31)

Abbreviations: NYHA: New York Heart Association functional class; Skin-AF: skin-autofluorescence; CML: carboxymethyllysine; CEL: carboxyethyllysine; eGFR: estimated glomerular filtration rate; LVEF: Left ventricular ejection fraction; E/A ratio: ratio between the peak early (E) and late (A) diastolic filling velocities; EE': ratio between the peak early diastolic filling (E) and the average early diastolic tissue velocity(E'); E': early diastolic tissue velocity; DCt: deceleration time; IVRT: isovolumetric relaxation time; ACE: angiotensin converting enzyme.

Baseline systolic and diastolic blood pressure were 158±17 mmHg and 91±10 mmHg. Baseline skin-AF was 2.4±0.4 a.u., while baseline levels of CML, CEL, and pentosidine were 1.5±0.3 µmol/l, 1.5±0.4 µmol/l, and 0.16±0.09 µmol/l, respectively. There were no significant differences in baseline characteristics between treatment groups [data not shown].

At the end of study in 7 out of 47 patients eprosartan was stopped. Blood pressure was reduced from 157/91 to 145/84 mmHg (P<0.001) in the eprosartan group and

from 158/91 to 141/83 mmHg (P<0.001) in the control group. Changes in systolic (-13±19 mmHg vs. -16±17 mmHg; P=0.74) and diastolic blood pressure (-7±10 vs. -7±10 mmHg; P=0.38) were not significantly different between the eprosartan and control group. Furthermore, changes in blood pressure were not different between patients with AGE levels above the median when compared to patients with levels below the median (data not shown).

The effects of eprosartan on AGE-accumulation are depicted in figure 1. Eprosartan use had no significant effects on changes in skin-AF (-0.06±0.3 vs. -0.07±0.3 a.u.; P=0.92), CML (-0.06±0.5 vs. 0.01±0.1 µmol/l; P=0.90), CEL (0.01±0.7 vs 0.05±0.6 µmol/l; P=0.80), and pentosidine (-0.025±0.1 vs. 0.008±0.1 µmol/l; P=0.11). Thus, overall, no effects of eprosartan on both plasma and tissue AGEs were found. In the whole group, CEL levels showed a small increase (1.47±0.39 vs. 1.58±0.46; P=0.01), while skin-AF and levels of CML, and pentosidine remained unchanged (P=0.27; P=0.62; P=0.62; respectively). Despite a large reduction in blood pressure, no effects were found with either eprosartan or control anti-hypertensive therapy on diastolic function (data not shown).

The second aim of the study was to relate baseline plasma and tissue AGEs to the response of blood pressure therapy on diastolic function. Table 2 depicts the relation

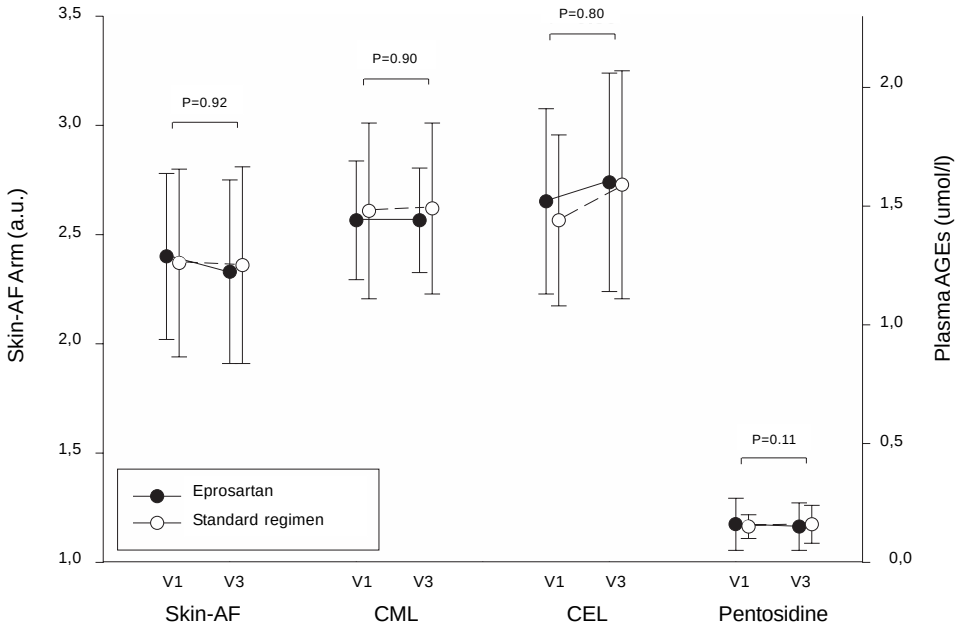

Figure 1. Changes in AGE-accumulation stratified for treatment group

Figure 1 depicts changes in AGE-accumulation stratified for treatment group. P-values denote the differences of the changes in AGE-accumulation among treatment groups calculated with student's t test or Mann-Whitney U test where applicable. Abbreviations: AGEs: advanced glycation end-products; Skin-AF: skin-autofluorescence; CML: carboxymethyllysine; CEL: carboxyethyllysine. Depicted are mean values with standard deviations.

Table 2. Correlations between baseline AGE levels and changes in diastolic function

Diastolic Function	R baseline AGEs			
	Skin-AF	CML	CEL	Pent
Delta				
E/A ratio	- 0.41*	- 0.02	- 0.06	0.12
IVRT	- 0.16	0.16	- 0.06	- 0.00
DCt	0.12	- 0.102	- 0.011	- 0.15
E'	- 0.46**	0.01	0.00	0.11
EE'	0.13	- 0.03	- 0.04	- 0.05
6 months				
E/A ratio	- 0.43*	0.07	- 0.00	0.05
IVRT	- 0.13	0.11	- 0.15	- 0.02
DCt	0.22	- 0.13	0.07	- 0.24*
E'	- 0.59***	0.05	- 0.16	- 0.08
EE'	- 0.02	- 0.12	0.00	- 0.05

*Abbreviations: R: correlation coefficient; Skin-AF: skin-autofluorescence; CML: carboxymethyllysine; CEL: carboxyethyllysine; Pent: Pentosidine; EE': ratio between the peak early diastolic filling (E) and the average early diastolic tissue velocity(E'); E': early diastolic tissue velocity; *=P<0.05; **=P<0.01; ***=P<0.001.*

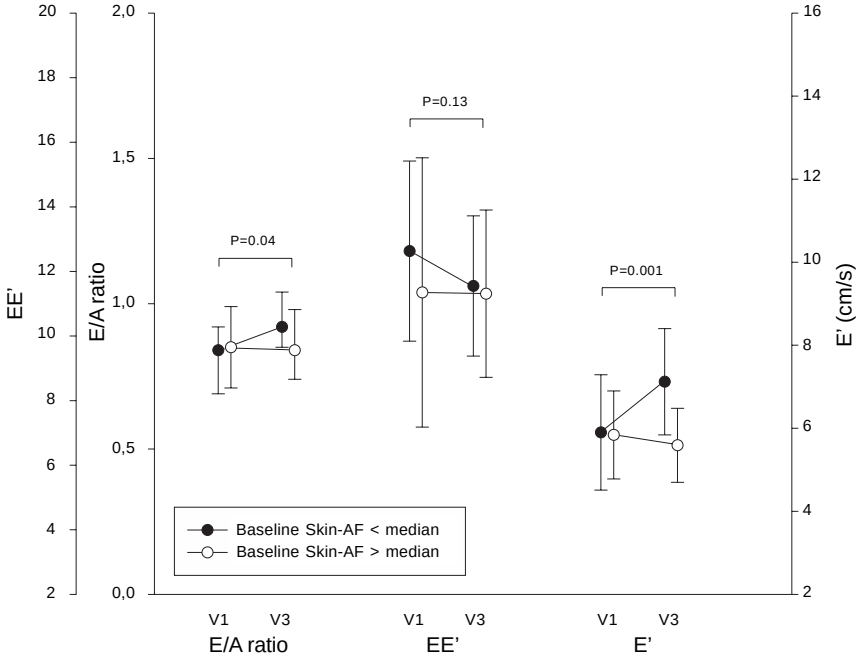

Figure 2. Changes in diastolic function stratified for baseline skin-AF

Figure 2 depicts changes in diastolic function stratified for baseline skin-AF. P-values denote the differences of the changes in diastolic function in the skin-AF group < median calculated with a paired student T-test or a Wilcoxon signed ranks test as appropriate. Abbreviations: E/A ratio: ratio between the peak early (E) and late (A) diastolic filling velocities; EE': ratio between the peak early diastolic filling (E) and the average early diastolic tissue velocity(E'); E': early diastolic tissue velocity. Depicted are mean values with standard deviations (E' and EE') and median values with 25%-75% interquartile range (E/A ratio) as appropriate.

between baseline AGE levels and changes in diastolic dysfunction. The level of baseline plasma AGEs were not related to changes in diastolic function. In contrast however, baseline tissue AGEs were related to a response on therapy. Table 2 shows that lower skin-AF at baseline was related with an improvement in E/A ratio (P=0.023) and an improvement in mean tissue E' (P=0.01).

Figure 2 shows a graphic representation of the relation between baseline skin-AF (> median vs. < median) and diastolic function at baseline and after 6 months of therapy. In patients with baseline skin-AF < median E/A ratio (from 0.84 [0.69-0.92] to 0.92 [0.85-1.04], P=0.04) and E' (from 5.9±1.4 to 7.1±1.3 cm/s, P=0.001) improved compared with patients with skin-AF levels > median in whom E/A ratio (from 0.85 [0.71-0.99] to 0.84 [0.74-0.98], P=0.84) and E' (from 5.8±1.1 to 5.6±0.9 cm/s, P=0.30) remained unchanged. A trend existed for an improvement of EE' in patients with skin-AF levels < median (from 12.6±2.8 to 11.6±2.2 cm/s, P=0.13) compared with patients with skin-AF levels > median (from 11.3±4.2 to 11.3±2.6 cm/s, P=0.96). No significant differences between patient with skin-AF < median compared with skin-AF > median were observed for DCT (P=0.71 vs. P=0.61, respectively), and IVRT (P=0.48 vs P=0.32, respectively). Using multivariable linear regression analysis we further validated our results by correcting for possible confounders. The significant relations that were found for skin-AF with change in E' (P=0.04) and change in E/A ratio (P=0.05) persisted after correction for age, renal function (eGFR), and the presence of diabetes.

Discussion

The results of the current study indicate that neither the angiotensin II type 1 receptor blocker (ARB) eprosartan nor control anti-hypertensive treatment decreased levels of plasma and tissue AGEs in patients with hypertension and diastolic dysfunction. Interestingly, however, we showed that in patients with lower skin-AF at baseline, diastolic function improved, in contrast to a lack of improvement in those with a skin-AF level above the median.

ARBs have shown the ability to lower in vitro and in vivo AGE formation and are thought to do so mainly by preventing the production of reactive carbonyl and dicarbonyl compounds (RCOs), which are critical precursors of AGEs.[7-9] However, conflicting data of available clinical studies on the effects of ARBs on AGE-accumulation have been presented. While two smaller studies by Saisho et al.[8] and Monacelli et al.[9] found that ARBs lowered plasma AGEs, two larger trials showed no effects of ARBs on AGE-accumulation.[10,11] It is therefore likely that ARBs at the dose used in the clinical situation (significantly lower than that for the in vitro studies) do not provide sufficient inhibition on plasma and tissue AGE formation. However, a possible treatment effect of ARBs may also have been overseen by the fact that 44% of our patients were using an ACE-inhibitor at study entry.

Patients with lower tissue skin-AF at baseline showed a significant improvement of diastolic function as a response to anti-hypertensive therapy, while patients with higher skin-AF did not. One explanation might be that in patients with higher skin-AF, more

AGE cross-links have been formed in the myocardium, which cannot be influenced by the current anti-hypertensive therapy. In contrast, in patients with lower skin-AF, less AGE cross-links may be present and the heart still has the ability to relax. Thus, skin-AF may be used to identify patients in whom an effect of blood pressure reduction on diastolic function can be expected.

This indicates the need for agents that can breakdown myocardial AGE cross-links to improve diastolic function. Such agents are AGE cross-link breakers. Preliminary data from two small intervention trials with the AGE cross-link breaker Alagebrium (ALT-711) have shown promising results in patients with chronic heart failure.[17,18] In both trials ALT-711 led to an improvement of diastolic function, results that warrant further investigations using AGE lowering therapies in the treatment of diastolic dysfunction and/or heart failure. A prospective randomised, double-blind, placebo-controlled trial on the effects of alagebrium on exercise tolerance and diastolic function in 100 chronic heart failure patients is currently ongoing (BENEFICIAL trial, www.clinicaltrials.gov; NCT00516646).

In contrast with tissue AGE-accumulation, plasma AGE levels were not associated with diastolic function in the present study. Both the LC-MS/MS method and the high performance liquid chromatography method used in our study to assess plasma AGEs are currently considered as the most accurate methods available. In our opinion, our findings may suggest that plasma AGE levels do not adequately reflect tissue AGE-accumulation. Furthermore, it would seem reasonable to assume that tissue AGEs are more closely related to diastolic function, because they are intrinsically linked with the actual patho-physiologic effects of AGEs (i.e., protein cross-linking). However, we cannot exclude the possibility that a power issue may explain the lack of correlation found between plasma AGEs and diastolic function.

One limitation of our study is the fact that skin-AF was only measured in a sub-population and therefore these results should be interpreted with caution. Also, although the autofluorescence measurements have been validated with tissue AGEs in the skin,[13,14] the correlation between skin-AF and AGEs in the myocardium has so far not been studied. Therefore, the present results are only hypothesis generating, and should be confirmed in a larger prospective study.

Conclusion

The angiotensin II type 1 receptor blocker eprosartan did not decrease levels of AGEs in patients with hypertension and diastolic dysfunction. However, irrespective of what anti-hypertensive drugs were used, patients with lower skin-AF at baseline showed a larger improvement in diastolic function in response to blood pressure reduction compared to those with higher skin-AF levels. Clinical trials using AGE lowering therapies are warranted to further explore the role of AGEs in the development and progression of diastolic dysfunction and subsequent heart failure.

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Chapter 4

Clinical and prognostic value of advanced glycation end-products (AGEs) in chronic heart failure

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Abstract

Introduction

Advanced glycation end-products (AGEs) have been proposed as a novel factor involved in the development and progression of chronic heart failure (CHF). We aimed to determine whether plasma levels of N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL), two well-known AGEs, are related to the severity and prognosis of CHF.

Methods

A total of 102 CHF patients, aged 58±12 year, with an average left ventricular ejection fraction of 28±9% were followed for 1.7[1.2-1.9] years. NYHA functional class and NT-proBNP were used as estimates of the severity of CHF. CML and CEL were determined by LC-MS/MS.

Results

CML levels were associated with NYHA functional class (P<0.001) and NT-proBNP levels (P<0.001). Survival analysis for the combined end-point of death, heart transplantation, ischemic cardiovascular event, and hospitalization for heart failure, revealed that CML levels predicted outcome, even after adjustment for age, gender, aetiology of CHF, identified risk modifiers, and several known predictors of outcome in CHF. The predictive value of CML subsided after correction for renal function. CEL was not associated with the severity or prognosis of CHF.

Conclusions

Plasma AGEs, in particular CML levels, are related to the severity and prognosis of CHF. The fact that the relation between CML and prognosis subsided after correction for renal function may suggest that AGE-accumulation in renal failure explains part of the prognostic value of renal function in CHF. However, further investigation is warranted to exclude the possibility that CML is just an innocent marker of renal function.

Introduction

Chronic heart failure (CHF) poses a significant burden to patients, health care providers, and society. Although mortality rates in CHF patients improved over the years, still roughly 50% of patients die within 5 years from diagnosis.[1] Several factors have been established as independent predictors for survival in CHF patients, among which are left ventricular ejection fraction (LVEF), NYHA functional class, and renal function.[2,3] In the last few years, advanced glycation end-products (AGEs) have received attention, since they may play a role in the pathophysiology of CHF.

AGEs are end-products of a non-enzymatic reaction of sugar and lipid adducts with proteins called the Maillard reaction.[4] AGEs form cross-links with long-living tissue proteins, which cause them to accumulate in the body with age.[5,6] AGE-accumulation in vivo is found throughout the body, including in skin, neural, vascular, renal, and cardiac tissue.[5,6] Enhanced accumulation is found in the presence of diabetes and renal failure.[5,7] Additionally, AGE precursors in cigarette smoke and food products are possible sources for increased AGE-accumulation.[8,9] AGE formation affects the physiological properties of proteins in the extracellular matrix, such as turnover, and elasticity.[10] Stimulation of AGE-receptors, such as the RAGE receptor, by AGEs leads to (prolonged) cellular activation and release of inflammatory cytokines.[10] These changes may result in the development and progression of diastolic and systolic dysfunction, and subsequent CHF.[11,12]

While AGE-accumulation has been primarily studied in patients with diabetes and renal failure, the clinical and prognostic value of AGEs in patients with CHF remains unkown. We determined whether plasma levels of N^{e}-(carboxymethyl)lysine (CML) and N^{e}-(carboxyethyl)lysine (CEL), two well-known AGEs, are related to the severity of CHF and prognosis.

Methods

Patients and study design

Patients and study design have been previously described by Smilde et al.[13] Stable CHF patients aged ≥18 years, with LVEF ≤ 45% were asked to participate. All patients had to have an optimal treatment for CHF, including at least a renin-angiotensin system inhibitor. Drug therapy had been stable for at least 1 month. Exclusion criteria were a myocardial infarction within the last 3 months, cardiac surgery or angioplasty within the last 3 months (or scheduled to undergo these procedures), unstable angina pectoris, primary renal disease, prior organ transplantation, or chronic use of renal function compromising medication. Special care was taken to include patients over the full range of severity of CHF. Approximately 121 patients were asked to participate. Between November 2003 and July 2005 in total 110 patients were included in the original analysis by Smilde et al.[13]

Samples for CML determination were unavailable in 8 patients, which left 102 patients eligible for the present analysis. All patients where Caucasian, except from 1 patient who was Black.

Baseline measurements included creatinine clearance and albumin excretion from 24-hours urine collections, glomerular filtration rate (GFR), effective renal plasma flow (ERPF), and extra cellular volume (ECV) measured as the clearance and the distribution volume of constantly infused ^{131}I-Hippuran and ^{125}I-iothalamate,[14] left ventricular ejection fraction (LVEF) measured by radionuclide ventriculography, NT-proBNP measured by electrochemiluminescence immunoassay, and levels of plasma CML and CEL by LC-MS/MS. Estimated GFR (eGFR) was calculated using the MDRD formula as described by Smilde et al.[13] The severity of CHF was classified in accordance with the NYHA functional class. A combined clinical outcome parameter was defined as the first occurrence of either death, heart transplantation, ischemic cardiovascular event (myocardial infarction or primary PTCA), or hospitalization for heart failure. Follow-up data was based upon the patients records available at our out-patient clinic. All patients routinely visited our clinic for heart failure treatment. When necessary information was gathered from local general practitioners. None of the patients were lost to follow-up. Median follow-up time of event-free patients was 1.7[1.2-1.9] years (range 1.0-2.4 years). Median time to first event was 0.7[0.2-1.1] years (range 0.02-1.56 years). This study protocol was approved by the institutional review committee of the University Medical Center Groningen. All patients signed written informed consent.

Plasma N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxyethyl)lysine by LC-MS/MS

Plasma N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl) lysine (CEL) were determined by stable-isotope dilution tandem mass spectrometry (LC-MS/MS) as described previously.[15] In short, 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 µmol/L nonafluoropentanoic acid as ion-pairing agent. Mass transitions of 205.1 -> 384.1 and 219.1 -> 384.1 for CML and CEL, respectively, and 209.1 -> 388.1 and 223.1 -> 388.1 for their respective internal standards were monitored in positive-ion mode. CML and CEL were separated by baseline resolution with a total analysis time of 21 min. Within-day and between-day coefficients of variation were <4.4% and <3.2% for CML, and <6.8% and <7.3% for CEL.

Statistical analyses

Data were analysed using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean±SD or as median [25-75% interquartile range] where applicable. Nominal variables are expressed as n(%). *P*-values for trend were determined by linear regression for continuous variables, and by χ^2 and Jonckheere–Terpstra tests for nominal and ordinal variables, respectively. By calculating *P*-values for trend over quartiles of CML and CEL we first identified factors that may influence the prognostic value of CML and CEL (risk-modifiers). Multivariable linear regression analysis was then used on all variables that showed a *P*-value of at least 0.10 or smaller in trend analysis to determine the variables that showed the strongest

association with CML and CEL. Variables which not retained significance in this multivariable analysis were subsequently removed from the model (backward selection). To test whether the model is appropriate and whether the assumptions for linear regression are met, the model has been tested for overall regression, co-linearity, interaction terms and lack-of-fit with ANOVA. Residuals were tested for normality of distribution. No violations were found. Next, we used Cox regression analysis to evaluate the prognostic value of CML and CEL. After basic adjustments of HR for age, gender, and aetiology of CHF, we made additional corrections for all risk-modifiers that showed a *P*-value of at least 0.10 or smaller in trend analysis. To further validate our model we made adjustments for several known predictors of outcome. Linearity of the continuous variables with respect to the response variable was assessed by determining the quartiles of their distribution. Subsequently, hazard ratios for each quartile were calculated. All variables showed a linear trend in the estimated hazard ratios, and were thus introduced in the model as continuous. Log-Minus-log survival curves and time-dependent covariates were used to evaluate adherence of the Cox proportional hazard assumptions. No violations of the proportional hazard assumption were identified. A *P*-value ≤0.05 (two-sided) was considered statistically significant.

Results

Baseline

Baseline characteristics are presented in table 1. We examined 102 CHF patients (77% male), aged 58±12, with a mean LVEF of 28±9%. Ischemic heart failure was present in 47% of patients. Cases of non-ischemic heart failure (54%) were most often patients with an idiopathic dilated cardiomyopathy (69%). Diabetes was present in only a small proportion (9%) of patients, as was a history of hypertension (16%).

Trend analysis revealed that higher CML levels were significantly associated with higher NYHA functional class ($P<0.001$), higher log NT-proBNP ($P<0.001$), older age ($P=0.004$), less smoking ($P=0.04$), lower GFR ($P<0.001$), increased BMI ($P=0.003$), and higher ECV (0.04). Additionally, a trend existed for an association between CML and lower diastolic blood pressure ($P=0.06$). Multivariable linear regression analysis showed that GFR ($P<0.001$) was the most important determinant of CML. Similar analysis revealed that higher CEL levels were significantly associated with older age ($P=0.03$), less smoking ($P=0.01$), lower diastolic blood pressure ($P=0.05$), lower GFR (0.005), and the use of β-blockade ($P=0.008$). A trend for an association existed between higher CEL and history of hypertension ($P=0.08$), and a higher NYHA functional class ($P=0.06$). The most important determinants of CEL levels were GFR ($P=0.004$), smoking ($P=0.02$), and use of β-blockade ($P=0.009$). Figure 1 illustrates the relation of NYHA functional class with CML and CEL. Figure 2 depicts a scatter plot of NT-proBNP levels versus CML and CEL levels.

Table 1. Baseline characteristics

Characteristic	Total (n=102)
Age (years)	58 ± 12
Sex (male)	78 (77)
Diabetes Mellitus, *n* (%)	9 (9)
History of hypertension, *n* (%)	16 (16)
Smoking, *n* (%)	16 (16)
Hypercholesterolemie, *n* (%)	55 (54)
History of CVD, *n* (%)	59 (58)
Body mass index (kg/m2)	27 ± 4
Systolic blood pressure (mmHg)	119 ± 21
Diastolic blood pressure (mmHg)	69 ± 12
Heart rate (bpm)	65 ± 13
Aetiology of CHF	
Ischemic, *n* (%)	47 (46)
Non-Ischemic, *n* (%)	
Idiopathic dilated cardiomyopathy	37 (36)
Post viral cardiomyopathy	4 (4)
Heart valve disease	3 (3)
Post partum cardiomyopathy	2 (2)
Alcoholic cardiomyopathy	1 (1)
Hypertension	1 (1)
Other	6 (6)
LVEF (%)	28 ± 9
NYHA functional class, *n* (%)	
I	14 (14)
II	47 (46)
III	31 (30)
IV	10 (10)
Medication use, *n* (%)	
ACEi/ARB	102 (100)
β-blockers	86 (84)
Diuretics	71 (70)
Calcium antagonists	13 (13)
Anti-arrhythmic	20 (20)
NT-proBNP (pg/ml)	634 [272-1849]
Creatinine (mmol/l)	104 [91-121]
Creatinine clearance (ml/min)	82 ± 34
eGFR with MDRD (ml/min)	63 ± 19
GFR (ml/min/1.73m^2)	75 ± 27
ERPF (ml/min/1.73m^2)	275 ± 88
Urinary albumin excretion (mg/24h)	9.3 [6.6-18.6]
ECV (L/kg body weight)	0.26 ± 0.05
CEL (μmol/l)	1.5 ± 0.5
CML (μmol/l)	1.7 ± 0.5

Note. Parametric parameters are expressed as mean ± SD; non-parametric parameters are expressed as median (25–75% IQR); ordinal parameters are expressed as n (%). Abbreviations: ACEi: Angiotensin converting enzyme inhibitors; ARB: Angiotensin II receptor blockers; BPM: Beats per minute; CHF: Chronic heart failure; CVD: Cardiovascular disease; ECV: Extra-cellular volume; GFR: glomerular filtration rate; LVEF: left ventricular ejection fraction; NT-pro-BNP: N-terminal-pro-brain natriuretric peptide; NYHA: New York Heart Association.

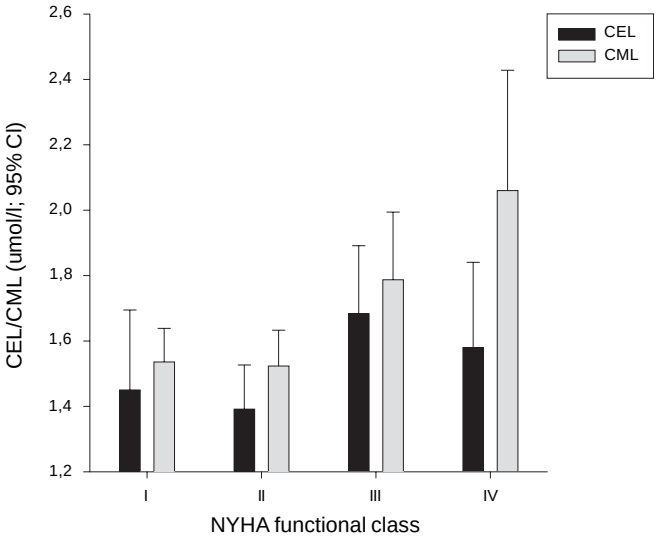


Figure 1. CML/CEL and NYHA functional class

Figure 1 depicts CML and CEL levels in plasma divided over NYHA functional class. Error bars indicate 95% confidence intervals. Trend analysis revealed that CML levels significantly increase with NYHA functional class (P<0.001). No significant differences were observed for CEL levels over NYHA functional class (P=0.06).

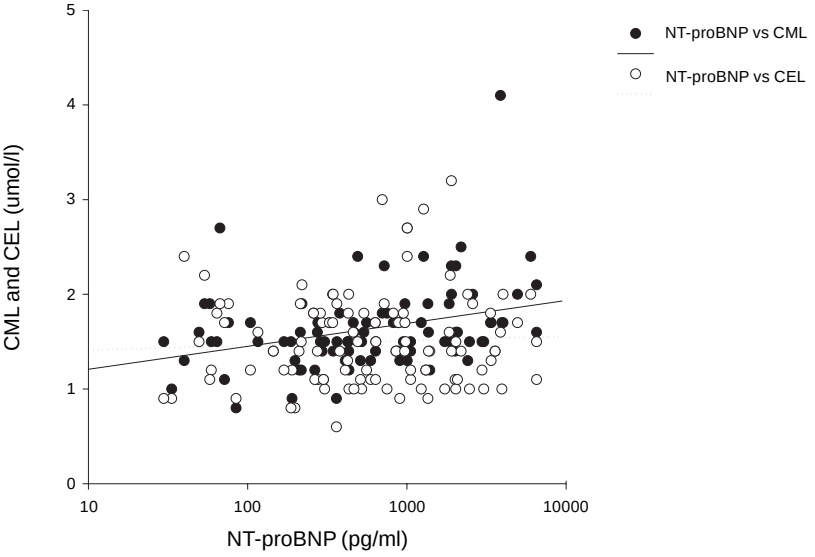


Figure 2. CML/CEL and NT-proBNP

Figure 2 depicts a scatter plot of CML and CEL levels in plasma versus log NT-proBNP levels. CML levels were significantly correlated with log NT-proBNP (r=0.40, P<0.001). No significant correlation was observed for CEL levels with log NT-proBNP (r=0.07, P=0.47).

Follow-up

Median follow-up for event-free patients was 1.7 [1.2-1.9] years (range 1.0-2.4 years). Twenty patients reached the combined end-point, of whom 6 died, 1 underwent HTx, and 13 patients were hospitalized for heart failure. None of the patients had an ischemic cardiovascular event. Figures 3a and b depict the results of Cox regression analysis. Univariate Cox regression analysis revealed that CML was a significant predictor of

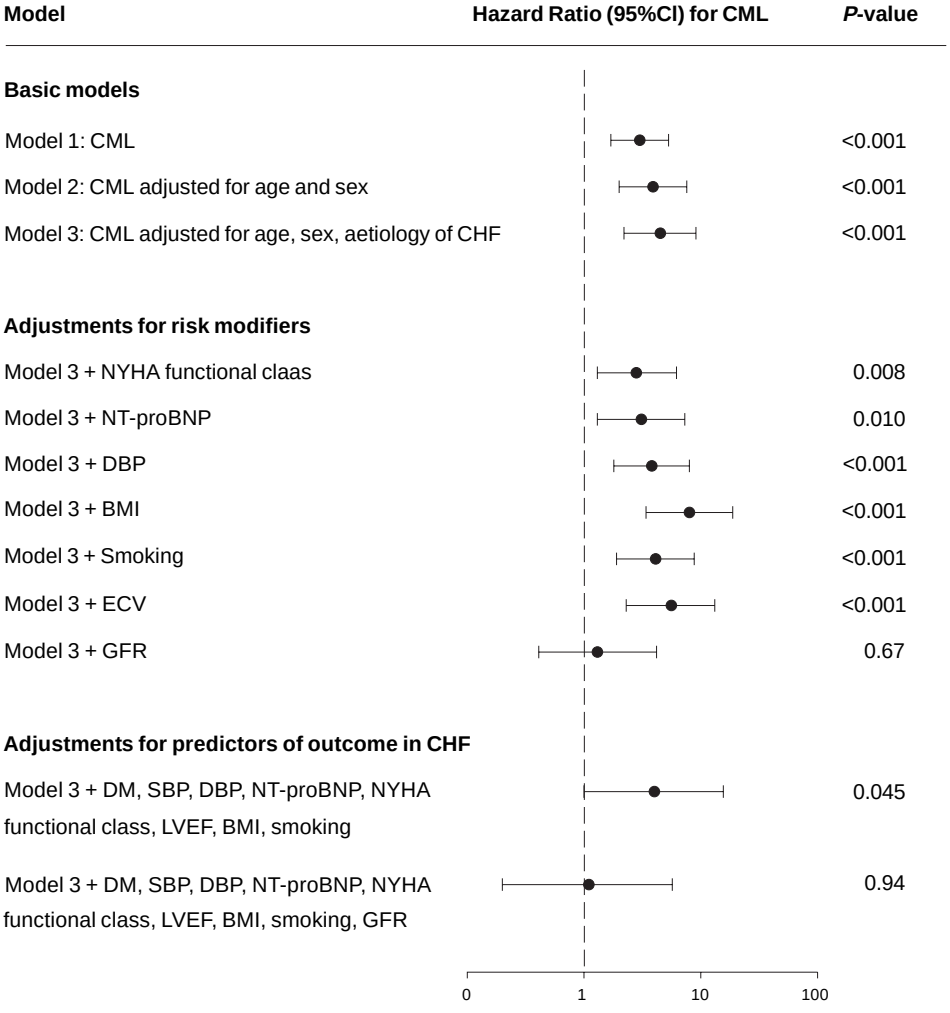


Figure 3a. Survival analysis of CML for the combined endpoint

Figure 3a depicts the results from the survival analysis of CML for the combined endpoint. First, basic adjustments were made for age, sex, and aetiology of CHF. Second, the relation between CML and the combined endpoint was adjusted for risk-modifiers identified with trend analysis, including NYHA function class, NT-proBNP, diastolic blood pressure (DBP), body mass index (BMI), smoking, extracellular volume (ECV), and glomerular filtration rate (GFR). Third, our basic model was corrected for important predictors of outcome in CHF, namely diabetes mellitus (DM), systolic blood pressure (SBP), DBP, NT-proBNP, NYHA functional class, left ventricular ejection fraction (LVEF), BMI, smoking, and GFR.

the combined endpoint. The prospective value of CML persisted after basic adjustments were made for age, sex and aetiology of CHF. Next, we made additional corrections for all risk-modifiers that showed a p-value of at least 0.10 or smaller in trend analysis. The relation between CML levels and outcome remained significant after adjustments were made for NYHA functional class, NT-proBNP, diastolic blood pressure, smoking, and ECV. However, the predictive value of CML subsided after adjustments for GFR

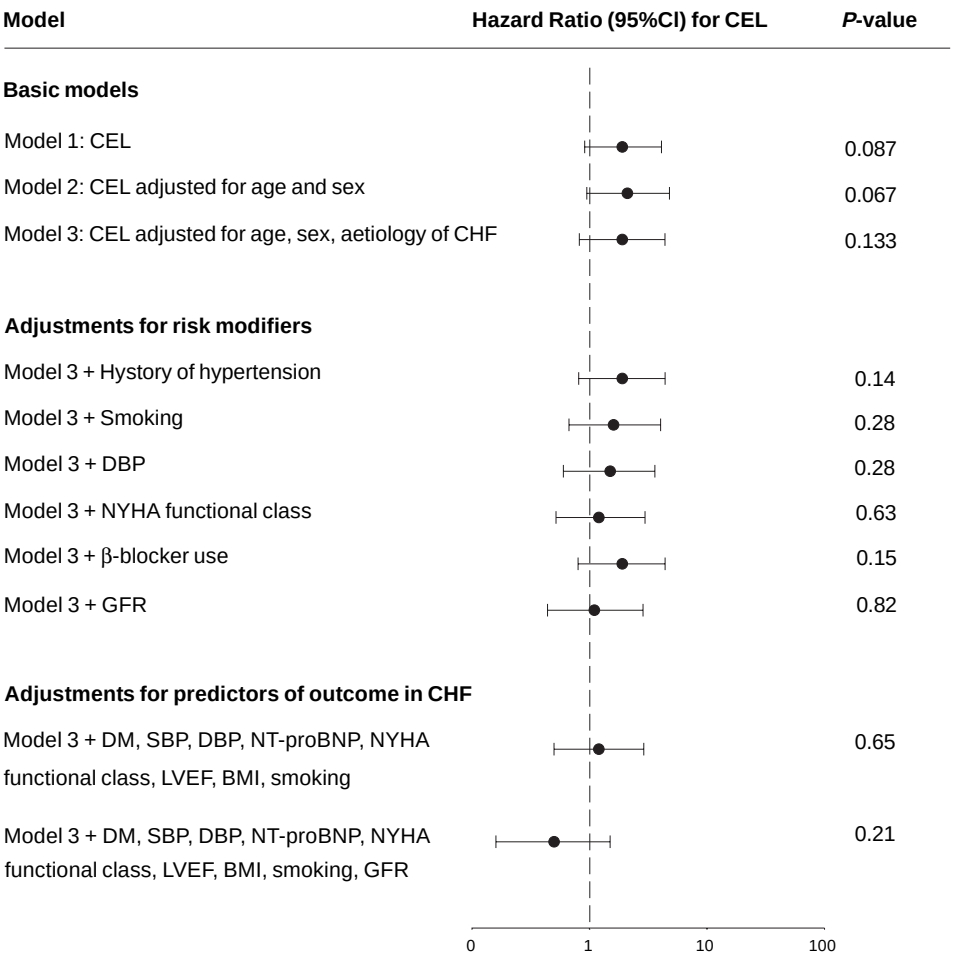


Figure 3b. Survival analysis of CEL for the combined endpoint

Figure 3b depicts the results from the survival analysis of CEL for the combined endpoint. First, basic adjustments were made for age, sex, and aetiology of CHF. Second, the relation between CEL and the combined endpoint was adjusted for risk-modifiers identified with trend analysis, including history of hypertension, smoking, diastolic blood pressure (DBP), β-blocker use, and glomerular filtration rate (GFR). Third, our basic model was corrected for important predictors of outcome in CHF, namely diabetes mellitus (DM), systolic blood pressure (SBP), DBP, NT-proBNP, NYHA functional class, left ventricular ejection fraction (LVEF), body mass index (BMI), smoking, and GFR.

were made. To discriminate whether the latter results implies an effect of hypoperfusion secondary to a more compromised systolic LVF or an effect of actual renal damage we evaluated the effect of adjustments for ERPF and urinary albumin excretion as risk modifiers as well. Adding ERPF to our model in stead of GFR resulted in an HR ([95% CI], *P*-values) for CML of 2.2 ([0.9-5.5], *P*=0.099), while correction for urinary albumin excretion resulted in similar HR for CML (4.4[2.2-9.0], *P*<0.001). To further validate our model we also made adjustments for important predictors of outcome in CHF. CML remained an independent predictor of outcome after adjustments for diabetes mellitus (DM), systolic blood pressure (SBP), diastolic blood pressure (DBP), NT-proBNP, NYHA functional class, left ventricular ejection fraction (LVEF), body mass index (BMI), and smoking. Again, the predictive value of CML subsided after additional adjustment for GFR. Although a trend exists for CEL to be associated with outcome, it did not reach statistical significance. In additional analysis it remained insignificant, also after corrections for the risk-modifiers identified in trend analysis, and important predictors of outcome in CHF. The survival curves for the combined endpoint show a decrease in survival with higher quartiles of CML and CEL, respectively (figure 4a and b).

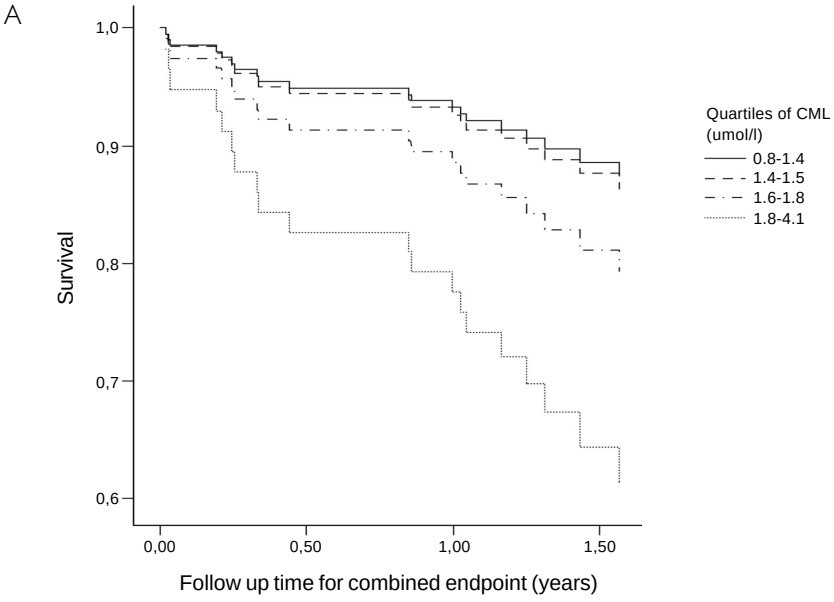
Baseline and follow-up results did not substantially change after correction of CML and CEL for plasma protein concentrations or extra cellular volume. Additionally, we evaluated the effect of other parameters of renal function as risk modifiers. Replacing GFR with creatinine, 24-hours creatinine clearance, and eGFR with MDRD, resulted in hazard ratio ([95% CI], *P*-values) for CML levels of 2.6([1.0-7.2]; *P*=0.05), 2.5([1.1-5.9], *P*=0.033), and 1.7([0.6-5.0], *P*=0.33), respectively.

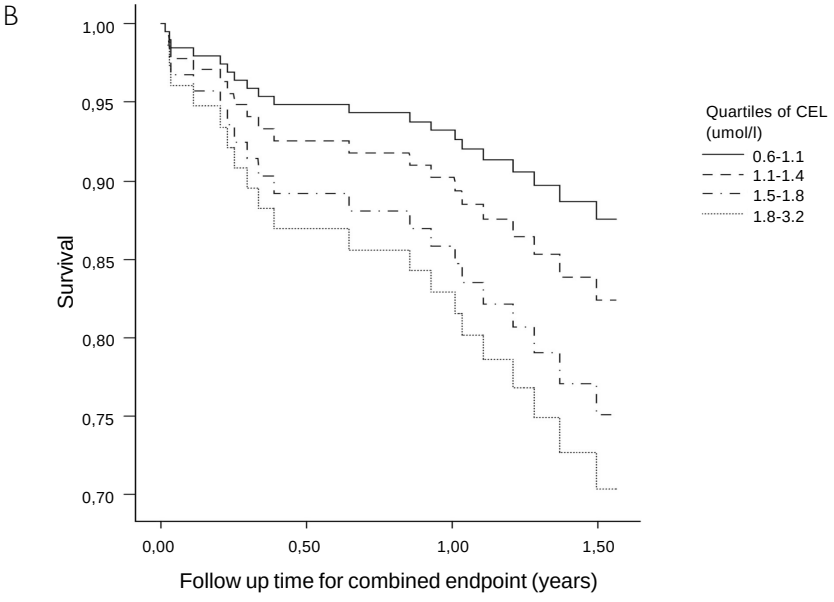
Discussion

The main finding of this study is that CML, a well-known AGE, is associated with the severity, and prognosis of patients with CHF. The relation between CML and outcome was independent of age, sex, aetiology of CHF, and identified risk modifiers, including NYHA functional class, NT-proBNP, diastolic blood pressure, body mass index, smoking, and ECV. Furthermore, it retained significance after adjustment for several known predictors of outcome in CHF. However, it subsided after adjustment for GFR. This is one of the first studies that analysed the relation between AGE levels and the severity and prognosis of CHF.

Several lines of evidence indicate that AGEs may play an active role in the development and progression of CHF. AGEs may be increased in CHF via the progression of renal failure, smoking, diabetes mellitus, age, and increased oxidative stress. Increased AGE-accumulation is associated with the development of diastolic and systolic dysfunction in experimental animal models.[16,17] Moreover, in preliminary human intervention studies, CHF patients seem to benefit from AGE breaking medication.[18,19]

The predictive value of CML found in this study was independent of age, sex, aetiology of CHF, identified risk-modifiers, and several predictors of outcome in CHF, but dependent of renal function. To validate this result we performed additional analysis using other parameters for renal function as risk modifiers. Although CML remained





Figures 4a and b. Survival for combined endpoint over quartiles of CML and CEL

Figures 4a and b show the survival for the combined endpoint over quartiles of CML and CEL. Survival curves were corrected for age and gender.

significantly associated with outcome after adjustment for creatinine, and creatinine clearance, adjustment for eGFR calculated using the MDRD formula resulted in more similar findings as for GFR. We feel that the latter result more reliably reflects the truth, a feeling which is strengthened by the results of Smilde et al,[13] who found that the MDRD formula is the most accurate indirect measurement of renal function in CHF.

Renal function is a clinically significant risk factor for mortality in CHF patients, independent of traditional prognostic factors, such as LVEF, and NYHA class.[3,20] AGEs are known to increase in renal failure due to decreased clearance.[21] Therefore, our results may indicate that CML levels explain (part of) the prognostic value of renal function in patients with CHF. This is further supported by our finding, that CML was not only related to prognosis, but also to a functional classification as well as a biochemical marker for the severity of CHF. However, due to the observational nature of our study, we cannot rule out the possibility that CML acts as a marker for impaired renal function, and as such might have predictive value in CHF.

By adding ERPF and urinary albumin excretion as risk modifiers to our model we made an attempt to discriminate whether the relations with GFR implies an effect of hypoperfusion secondary to a more compromised systolic LVF or an effect of actual renal damage. The fact that the HR for CML decreased substantially after adding ERPF to our model in stead of GFR, but not changed after addition of urinary albumin excretion suggests that hypoperfusion is far more likely than renal damage.

While the chemical structures of CML and CEL are quite similar, the results we obtained for both are not. One reason may be that CML originates from different pathways than CEL. CML can be formed through lipid peroxidation and glycoxidation pathways, while CEL is mainly formed through glycoxidation pathways.[22]

AGE levels were previously evaluated in CHF by Heidland et al.[23] They evaluated plasma AGEs in a small group of patients with severe CHF, heart transplant recipients, and normal controls. Paradoxically, they found a decrease in CML and AGE-fluorescence in patients with CHF when compared to controls. Heart transplant recipient did, however, show an increase in measured AGE data. Unfortunately, data on NYHA functional class, NT-proBNP levels, and prognosis were not provided. The authors suggested that their results were possibly biased by hypervolemia, lowered plasma protein concentrations, and decreased dietary intake of AGEs in CHF. While we cannot correct our data for dietary intake of AGEs, correction of our data for plasma protein concentrations did not substantially change our results. Moreover, correction for extra cellular volume (ECV) which could be estimated from the distribution volume of ^{125}I-iothalamate, did not change our results either.

Recently, Koyama et al[24] for the first time evaluated the prognostic value of serum AGEs as risk factor in CHF. They found that serum pentosidine levels were a significant predictor of cardiac death and re-hospitalization. Although they corrected their findings for other known risk factors in CHF, like BNP, renal function, age, and NYHA functional class they may have introduced a possible co-linearity problem by simultaneously introducing creatinine levels and estimated GFR in the multivariable model. Therefore, their data should be interpreted with caution.

The prognostic value of AGEs has been studied in other populations than CHF as well. Kilhovd et al[25] showed that high levels of circulating AGEs predicted cardiovascular mortality in non-diabetic women. However, they presented their data uncorrected for renal function, and their results may, therefore, be biased. In patients with renal failure results vary widely. While Schwedler et al[26] and Busch et al[27,28] reported that circulating AGE levels were not related to prognosis, Wagner et al.[29] and Roberts et al[30] did find prognostic value of circulating AGEs. Our group previously demonstrated that AGE-accumulation measured by skin-autofluoresence, was a strong and independent determinant of prognosis in both dialysis and diabetic patients.[31,32]

In many of the above mentioned studies, enzyme-linked immunosorbent assay (ELISA) was used to determine AGE levels. Several difficulties exist with standardization of ELISA methods to assess AGE levels. Therefore, the results of this studies should be interpreted with caution. In contrast, in the present study we used LC-MS/MS to determine CML and CEL levels, which is currently seen as the most accurate method to assess plasma AGE levels.

Conclusion

Plasma AGEs, in particular CML levels, are related to the severity and prognosis of CHF. The fact that the relation between CML and prognosis subsided after correction for renal function may suggest that AGE-accumulation in renal failure explains part of the prognostic value of renal function in CHF. However, further investigation is warranted to exclude the possibility that CML is just an innocent marker of renal function.

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Part 2

AGEs in Renal Failure

Chapter 5

Advanced glycation end-products in kidney transplant patients: a putative role in the development of chronic renal transplant dysfunction

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ABSTRACT

Chronic renal transplant dysfunction is one of the leading causes of graft failure in kidney transplantation. A complex interplay of both alloantigen-related and alloantigen-unrelated risk factors is believed to underlie its development. We propose that advanced glycation end-products (AGEs) are involved in the development of chronic renal transplant dysfunction. AGE-formation is associated with different alloantigen-unrelated risk factors for chronic renal transplant dysfunction, such as recipient age, diabetes, proteinuria, hypertension, and hyperlipidemia. In vitro studies have shown that AGEs induce the expression of various mediators associated with chronic renal transplant dysfunction. Furthermore, AGE-induced renal damage has been found in multiple experimental studies. This renal damage shows similarity to the damage found in chronic renal transplant dysfunction. Together, several lines of evidence support a role of AGEs in the development of chronic renal transplant dysfunction and suggest that preventive therapy with AGE inhibitors may be helpful in preserving renal function in transplant recipients.

Introduction

The development of new immunosuppressive drugs has improved short-term graft survival in kidney transplant recipients substantially.[1,2] Although overall long-term graft survival is improving slowly, it does not parallel improvements in short-term survival.[2] Approximately 60% of patients receiving cadaveric donor kidneys will develop graft failure within 10 years after transplantation.[1]

Chronic renal transplant dysfunction, also known as chronic allograft nephropathy, is one of the leading causes of late graft failure. Chronic renal transplant dysfunction is characterized clinically by a slow, but steady, decline in function of the transplanted kidney, associated with the development of hypertension and proteinuria.[3] Histopathologic characteristics of chronic renal transplant dysfunction include arteriosclerosis of the intrarenal vasculature, glomerulosclerosis, and interstitial fibrosis with tubular atrophy.[4] A complex interplay of both alloantigen-dependent and alloantigen-independent risk factors is believed to underlie the development of chronic renal transplant dysfunction.[3] Alloantigen-dependent factors include episodes of acute rejection, inadequate immunosuppression, and increased HLA mismatching.[3] Alloantigen-independent factors include recipient and donor age,[5] impaired renal function,[6] hypertension,[7] the presence of diabetes,[8] proteinuria,[9] hyperlipidemia,[10] obesity,[11] transplant ischemia,[12] and use of calcineurin inhibitors.[13] The extent of their contributions is largely unknown.

Interestingly, to a certain extent, alloantigen independent risk factors for the development of chronic renal transplant dysfunction overlap risk factors for the accumulation of advanced glycation end-products (AGEs). This overlap is well established for age,[14] renal function impairment,[15,16] and diabetes.[17] Although less conclusive, evidence exists that associates hypertension,[18,19] proteinuria,[20] and hyperlipidemia[21] with enhanced AGE-accumulation. This led us to believe that AGEs might be involved in the pathogenesis state of chronic renal transplant dysfunction. In this report, we summarize the evidence for a role of AGEs in the development of chronic renal transplant dysfunction. First, we discuss recent insights in AGE kinetics. Second, we discuss data on plasma and tissue AGE levels in patients with a kidney transplant. Third, we propose mechanisms through which AGEs may be involved in the development of chronic renal transplant dysfunction. Finally, we discuss studies on AGE-induced renal tissue damage.

AGE kinetics

Historically, AGEs have been considered end products from a nonenzymatic reaction between sugars and proteins, called the Maillard reaction.[22] The final steps in the Maillard reaction are driven by oxidative stress, defined as the steady state level of reactive oxygen species.[23] Because AGEs are able to accelerate oxidation strongly, they favor their own production.[23,24] Figure 1 shows classical and newly discovered pathways of AGE formation. Currently, it is known that some AGEs are derived from lipid peroxidation; therefore, advanced lipoxidation end products would be a better

name for this subgroup of AGEs. However, we use the term AGEs when referring to both AGEs and advanced lipoxidation end products. Furthermore, it was discovered that in addition to oxidative stress, carbonyl stress, ie, the steady-state level of reactive carbonyl compounds, is thought to be centrally involved in AGE formation.[25,26] Reactive carbonyl compounds are derived from the reaction of lipids or carbohydrates with reactive oxygen species. These compounds subsequently react with proteins to form AGEs and advanced lipoxidation end products. Examples of reactive carbonyl compounds include methylglyoxal and glyoxal.[25,26]

The formation and accumulation of AGEs in tissue, the amount of AGEs circulating in the bloodstream, and the excretion of AGEs by the kidney seem to be in dynamic equilibrium. AGEs form cross-links with long-living tissue proteins, which enable them to accumulate in the body.[27] AGE-accumulation in tissue is associated with aging,[14] renal function impairment,[28] and the presence of diabetes.[17] External sources of AGEs include AGE precursors in cigarette smoke and alimentary intake of AGEs.[29,30] Detoxification of AGEs depends on both the degradation of AGEs to AGE peptides by macrophages[31] and renal clearance of AGEs. There is evidence for filtration of AGE compounds through glomeruli and active reabsorption in proximal tubuli. After modification or degradation in proximal tubuli, AGEs eventually are cleared in urine.[32,33]

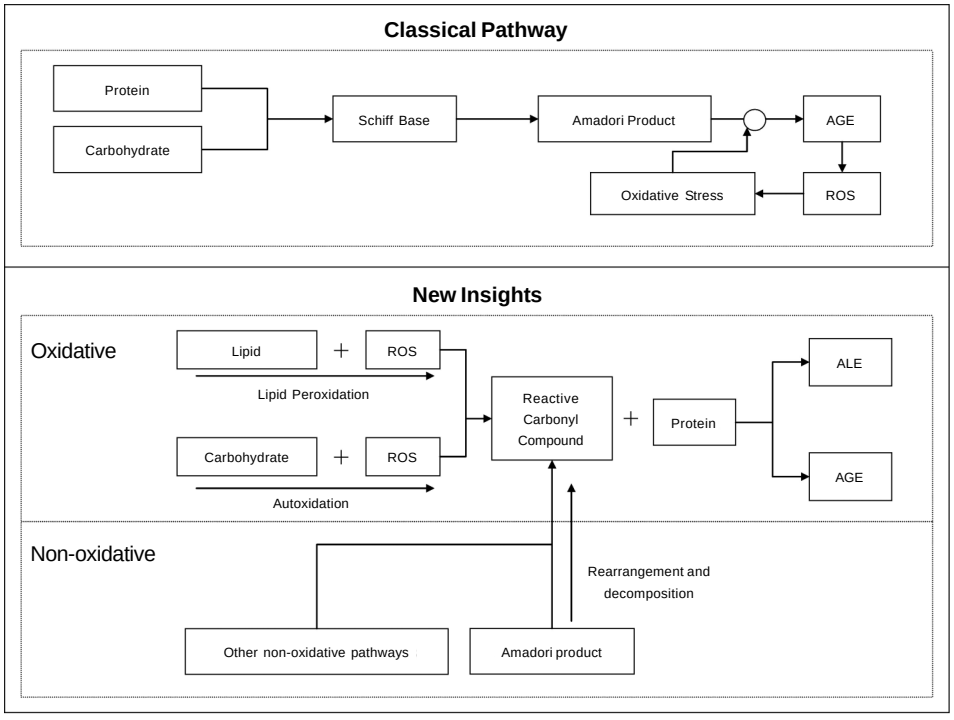

Figure 1. Classical pathway of AGE formation and new insights
Abbreviations: ROS, reactive oxygen species; ALE, advanced lipoxidation end product.

Although several methods to determine AGE-accumulation have been described, no commercial assay or tool is available yet. Classically, AGEs are determined by using their characteristic fluorescence properties.[34] Currently, gas chromatography mass spectrometry is considered the most accurate technique to determine AGE levels.[35] High-performance liquid chromatography also is accurate, but is relatively time consuming.[36] Several difficulties exist with standardization if an enzyme-linked immunosorbent assay is used.[37] Furthermore, fluorescent techniques have been adapted to enable their use in clinical studies.[38] In addition to biochemical assays and fluorescent techniques, several immunohistochemical techniques have been described to determine AGE levels.[39] One should consider differences in accuracy of the techniques used when interpreting data on AGE levels.

AGE-LEVELS IN KIDNEY TRANSPLANT PATIENTS

Before exploring AGE-accumulation in kidney transplant recipients, it is important to realize that most transplant recipients have experienced a long period of impaired renal function before transplantation. AGEs accumulate during the period of gradual renal function loss and during dialysis treatment.[28] Thus, kidney transplant recipients most often have high AGE levels before transplantation. AGE levels in transplant donors are unknown. Presumably, a wide variability in donor kidney AGE levels exists because of the heterogeneity of donors. However, it is reasonable to assume that donors will have lower tissue AGE levels than transplant recipients. Thus, a kidney with presumably low AGE levels is transplanted into an AGE-rich environment. Kidney transplantation aims to restore renal function and thereby is thought to lower AGE levels. Questions are to what extent AGE-accumulation will resolve after kidney transplantation and how the transplanted kidney behaves in an AGE-rich environment. Several research groups have investigated the influence of kidney transplantation on AGE levels in tissue and blood. Unfortunately, only data on extrarenal AGE levels have been published. No data are available on AGE levels in kidneys of transplant recipients, either with or without chronic renal transplant dysfunction. Thus, we do not know how the transplanted kidney handles the AGE-rich environment it is placed in. Although it is interesting to hypothesize that the transplanted kidney is more prone to AGE formation because of local proinflammatory stimuli, the current lack of data on renal AGE levels limits us to expand on this thought. The different studies on AGE levels in pretransplantation and posttransplantation patients are listed in table 1.

Blood AGE levels are increased strongly in patients on dialysis therapy compared with controls. Although transplantation reduces blood AGE levels, these generally remain greater than normal. Interestingly, studies evaluating blood AGE levels within the first 6 months after transplantation showed that blood AGE levels decreased by 70% to 80%. This suggests that a decrease in blood AGE levels occurs early after improvement of renal function.[40,41] Some investigators reported disproportionally high blood AGE levels after transplantation when related to renal function.[42,43] Thus, other factors not already present in patients with chronic renal insufficiency and unrelated to renal function may influence AGE formation after transplantation as well. One

Table 1. The effect of kidney transplantation on AGE-levels in tissue and blood

Reference	Study design		Pretransplantation*			Posttransplantation*			Post- vs. Pretransplantation		Remarks
	Endpoint	Method	Type	No. of Patients	Fold vs. Controls	No. of Patients	Time	Fold vs. Controls	Reduction (%)	*P*	
Data on blood AGEs											
Makita et al[40]	Se-AGE	RRA	HD	6	5.31	16	2-9 y	1.56	71	< 0.001	Diabetic
	Se-AGE	RRA	D	2	4.62	2	14 d	1.36	71	-	Prospective
Hricik et al[15]	Pl-Pent	HPLC	D	41	27.5	39	24 mo	3.3	88	< 0.05†	Prospective
Hricik et al[49]	Pl-Pent	HPLC	HD+PD	88	21.2	15	6-80 mo	2.2	89	0.002	Prospective
Miyata et al[41]	Pl-Pent-Alb	HPLC	HD	29	10.7	7	6 mo	1.5	86	< 0.05†	Prospective
	Pl-Pent-Alb	HPLC	HD	29	10.7	12	6.2 y	1.0	91	< 0.05†	
Sebekova et al[42]	Pl-Fluor	Sp	HD+PD	10	4.8	9	34 mo	2.4	50	< 0.01	Pediatric
	Pl-CML	ELISA	HD+PD	10	3.3	9	34 mo	3.0	15	Ns	Pediatric
Misselwitz et al[16]	Se-Pent	HPLC	PD+HD	9	16.4	12	0.5-6 y	2.4	85	< 0.01	Pediatric
	Se-CML	ELISA	PD+HD	9	2.2	12	0.5-6 y	1.0	53	< 0.01	Pediatric
Data on tissue AGEs											
Lee et al[46]	Skin-Ti-CLF	Sp	CRF	18	2.45	16	11 wk	1.26	49	0.003	Non-diabetic
	Perit-Ti-CLF	Sp	CRF	13	1.89	15	11 wk	1.20	37	Ns	Non-diabetic
Hricik et al[49]	Skin-Ti-Pent	HPLC	HD+PD	88	59.7±21.7 pmol/mg‡	15	6-80 mo	57.9±17.3 pmol/mg‡	3	Ns	Prospective
Yoshida et al[47]	Card-Ti-CML	IH	HD+PD	10	3.6	8	5.8 y	2.2	39	< 0.05	
	Card-Ti-AGE	IH	HD+PD	10	0.96	8	5.8 y	0.55	43	Ns	
Shaw et al[48]	Skin-Ti-CLF	Sp	CRF	26	11.7±4.51 a.u./mg‡	18	3-43 mo	5.0±3.13 a.u./mg‡	57	< 0.001	Non-diabetic
	Skin-Ti-Pent	HPLC	CRF	13	245.4±77 pmol/mg‡	9	3-43 mo	65.9±40 pmol/mg‡	73	< 0.001	Non-diabetic

Abbreviations: Perit, peritoneal; Card, cardial; Se, serum; Pl, plasma; Ti, tissue; Pent, pentosidine; CML, carboxymethyllysine; Alb, albumine; Fluor, fluorescent. AGEs; CLF, collagen linked fluorescent; RRA, radio receptor assay; HPLC, high-performance liquid chromatography; Sp, spectrometry, ELISA, enzyme-linked immunosorbent assay; IH, immunohistochemistry; HD, hemodialysis; PD, peritoneal dialysis; CRF, chronic renal failure; D, dialysis (non-specified); Ns, not significant.

**AGE levels in patients were divided by AGE levels in healthy controls to calculate fold difference from control; †Although differences were significant, significance levels were not provided; ‡No controls available, concentrations are given in picomoles per milligram or arbitrary units per milligram.*

explanation could be that enhanced AGE-accumulation in relation to renal function reflects an enhanced nutritional status.[44] Another explanation could be the use of calcineurin inhibitors, especially cyclosporine, in transplant recipients. Use of cyclosporine has been associated with enhanced oxidative stress and thus might be of influence on AGE levels found in kidney transplant recipients.[45]

Results of studies on the influence of kidney transplantation on AGE-accumulation in tissue are inconclusive. Although the idea that kidney transplantation decreases tissue AGE-accumulation is supported by some studies,[46-48] Hricik et al[49] showed that kidney transplantation does not correct tissue AGE-accumulation. They found an increase in tissue AGE levels in the majority of patients studied. Although there is reason to believe that a decrease in blood AGE levels eventually is reflected in a decrease in tissue AGE-accumulation, studies on tissue AGE levels are limited in number, size, and duration after transplantation (mostly <4 to 5 years). Few data currently are available on the kinetics of tissue AGE-accumulation in the long run after transplantation. Recently, data were published on extrarenal AGE levels in patients who developed chronic renal transplant dysfunction.[50] Patients with biopsy-proven chronic renal transplant dysfunction had greater AGE levels compared with transplant recipients with normal renal function and patients with chronic renal failure of their native kidneys. These findings argue that the increased AGE levels in patients with chronic renal transplant dysfunction cannot be attributed solely to the effect of decreased renal function in patients with chronic renal transplant dysfunction.

AGE-induced cellular responses

We wonder whether AGEs are innocent bystanders or contribute actively to the pathophysiological processes underlying the development of chronic renal transplant dysfunction. In figure 2, we propose a cascade of events that may be involved. It refers to cell types that express AGE receptors, mediators released in response to activation of these receptors, and tissue damage that resulted from those mediators in different in vitro experiments. AGE receptor expression has been found in a wide range of cells, such as endothelial cells,[51] monocytes,[52] macrophages,[31] mesangial cells,[53] smooth muscle cells,[54] and tubular cells.[55] The various cells release different mediators when stimulated by AGEs, inducing an inflammatory response that may lead to tissue damage.

Endothelial cells

Endothelial cells are thought to be centrally involved in the process of inflammation. Different inflammatory mediators are released after activation of receptors on endothelial cells. When stimulated by AGEs, endothelial cells release the inflammatory mediators vascular cell adhesion molecule-1 and intercellular adhesion molecule-1.[51,56] Release of these inflammatory mediators is influenced by oxidative stress and nuclear factor-κβ expression,[57-60] which are both enhanced by AGEs in vitro.[4,61] Oxidative stress is enhanced in patients with end-stage renal failure[62] and kidney transplant recipients.[63] In addition, it was shown that transplant recipients with chronic rejection experience significantly more oxidative stress than patients without chronic rejection.[63]

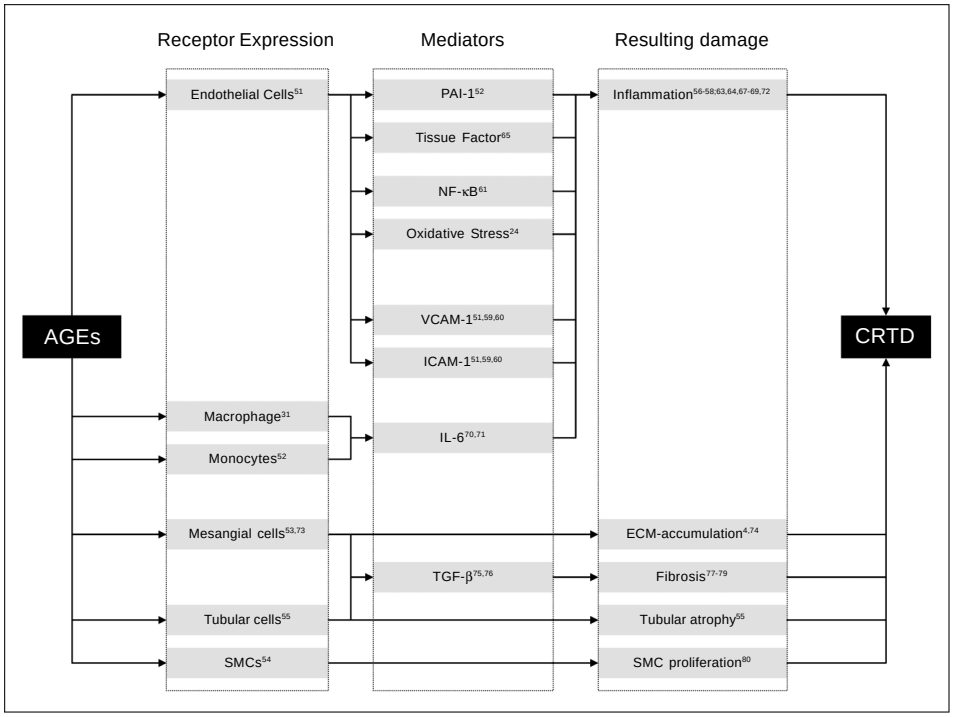

Figure 2. Effect of AGEs on different cell types involved in the development of chronic renal transplant dysfunction

Abbreviations: CRTD, chronic renal transplant dysfunction; PAI-1, plasminogen activator inhibitor 1; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin-6; NF-κB, nuclear factor-κB; ECM, extracellular matrix; TGF-β, transforming growth factor-β; SMC, smooth muscle cell.

Inflammation also has been associated with chronic rejection. An immunohistochemical study of transplant biopsy specimens showed enhanced expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in chronic rejection.[64] In endothelial cells, AGEs induce the production of tissue factor and plasminogen activator inhibitor-1, as well.[65,66] Tissue factor is the major cellular trigger of blood coagulation. Activated plasminogen activator inhibitor-1 inhibits the activation of plasminogen to plasmin, resulting in thrombosis.[67] In addition to their involvement in blood coagulation, plasminogen activator inhibitor-1 and tissue factor are thought to have important proinflammatory capabilities.[68,69]

Monocytes and macrophages

Monocytes and macrophages are actively involved in the inflammatory process after their attraction and activation by endothelial cells. Interleukin-6, produced by both cell types when stimulated by AGEs in vitro, stimulates the liver to produce acute-phase proteins.[56,70,71] In a rat model of chronic kidney allograft rejection, enhanced

interleukin-6 expression was associated with graft failure.[72] Furthermore, human monocytes stimulated by AGEs produce insulin-like growth factor, which is known to stimulate mesangial cells.[73]

Mesangial cells, smooth muscle cells, and tubular cells

In response to AGEs, mouse mesangial cells showed increased expression of collagen type IV messenger RNA, leading to accumulation of extracellular matrix.[74] Accumulation of extracellular matrix is one of the histological findings in chronic renal transplant dysfunction.[4] Furthermore, both mesangial cells and tubular cells stimulated by AGEs produce transforming growth factor-β.[75,76] Transforming growth factor-β mediates the formation of fibrosis.[77] Transforming growth factor-β expression in a renal allograft correlates with the development of interstitial fibrosis.[78] Moreover, increased transforming growth factor-β expression has been found in renal biopsy specimens of patients with chronic renal transplant dysfunction.[79] Finally, smooth muscle cell proliferation and tubular atrophy, both found in chronic renal transplant dysfunction, have been associated with AGE-accumulation.[55,80]

AGE-INDUCED RENAL TISSUE DAMAGE

Although several AGEs, such as pentosidine, N^{ε}-carboxymethyllysine, and N^{ε}-carboxyethyllysine, have been characterized, differences in the pathogenic role between specific AGEs are not yet clear. The pathogenic role of AGEs on renal tissue has been tested in various experimental studies. In a study by Vlassara et al,[81] 50 healthy male Sprague-Dawley rats were administered AGE modified rat albumin, native rat albumin, or AGE-modified rat albumin in combination with aminoguanidine. Repeated injections with AGEs resulted in increased AGE levels in blood and kidney. AGE-injected animals showed an increase in glomerular volume, glomerular basement widening, and mesangial extracellular matrix, indicating global and segmental glomerulosclerosis. These structural changes were less pronounced in rats administered aminoguanidine.[81] Furthermore, AGE injections resulted in increased total urinary protein excretion, which was almost completely prevented with aminoguanidine treatment. In another experiment by Vlassara's group, the effect of aminoguanidine on age-related renal pathological characteristics was examined. Nondiabetic female Sprague-Dawley rats and Fischer-344 rats were treated with aminoguanidine for 18 months. Aminoguanidine significantly decreased renal AGE-accumulation compared with nontreated controls. Moreover, aminoguanidine partly inhibited age-related albuminuria and proteinuria. In Sprague-Dawley rats, the age-related decrease in glomerular number, accompanied by progressive glomerular sclerosis, was significantly ameliorated by aminoguanidine treatment. In Fischer-344 rats, observed age-related changes were less pronounced. Consequently, no significant structural effects of aminoguanidine were found in this strain.[82] More recently, Vlassara's group tested whether a diet low in glycoxidation products could prevent diabetic nephropathy in mice.[83] Nonobese diabetic mice were randomly assigned to an AGE-rich or low-AGE

diet. Both serum and kidney AGE levels were significantly lower in the low-AGE diet group. Rats fed an AGE-rich diet developed progressive diabetic nephropathy and had short survival, whereas rats fed a low-AGE diet developed only minimal glomerular pathological characteristics and had a significantly extended survival. It remains questionable whether observed effects could be attributed to the toxicity of AGEs alone. One should consider possible effects of other toxic compounds formed under similar conditions as AGEs.[84] Furthermore, in relation to alimentary AGEs, antioxidant effects of some of the Maillard reaction products formed also should be anticipated.[85]

Results from Vlassara's group[81-83] are in line with those reported by others. Soulis-Liparota et al[86] examined the effect of aminoguanidine on the development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin induced diabetic rats during a 32-week period. Compared with untreated controls, aminoguanidine prevented diabetes-induced increased fluorescence in isolated glomeruli and renal tubules, but not in the whole kidney. Furthermore, aminoguanidine treatment attenuated the increase in albuminuria and mesangial expansion. The use of other AGE inhibitors in experimental studies, such as N-(2-acetamidoethyl)-hydrazinecarboximidamide-hydrochloride (ALT-946),[87] (±)-2-isopropylidenehydrazono-4-oxothiazolidin-5-ylacetanilide (OPB-9195),[88] and pyridoxamine,[89] has confirmed the results of studies mentioned. In conclusion, AGE-induced renal tissue damage is well established in both diabetic and nondiabetic animal models. Although observed changes are often nonspecific, they are similar to lesions observed in chronic renal transplant dysfunction. While heavily modified proteins were used in the first study described from Vlassara's group,[81] recent studies examined the effect of more clinically relevant age-related or diabetes-related increases in AGE-accumulation on renal tissue. To date, no results of experimental studies with AGE inhibitors have been published in chronic renal transplant dysfunction rat models. Moreover, no clinical trials have been performed in kidney transplant recipients using AGE-lowering treatment modalities.

Conclusion

We discussed evidence for a pathogenic role of AGEs in the development of chronic renal transplant dysfunction. First, AGE levels are elevated in the presence of some risk factors involved in the development of chronic renal transplant dysfunction. Although few data currently are available on the kinetics of tissue AGE-accumulation in the long run after transplantation, increased AGE levels were found in blood of patients who developed chronic renal transplant dysfunction. In vitro data showed that AGEs may stimulate various cells to release mediators that contribute to the renal damage found in chronic renal transplant dysfunction. Based on these findings, we proposed a pathophysiological mechanism of AGE-induced renal tissue damage. Finally, we discussed results of experimental studies on AGE-induced renal tissue damage. To date, no studies, experimental or clinical, have been performed to examine the effect of AGE-lowering treatment modalities on the development of chronic renal transplant dysfunction.

Opponents of AGE-related hypotheses argue that AGEs are detectable only in trace concentrations in tissue proteins and therefore could not be important pathogenic constituents. Proponents argue that new AGEs are still being discovered and little is known about AGE effector mechanisms. However, various studies have associated AGE-accumulation with vascular disease processes. Together, these studies illustrated the pathogenic potential of AGEs in vitro and in vivo. We expect clinical studies to confirm the role of AGEs in the development of chronic renal transplant dysfunction. In the future, therapy with AGE-formation inhibitors or AGE crosslink breakers may be warranted to preserve renal function in transplant recipients.

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Chapter 6

Accumulation of advanced glycation end-products, measured as skin-autofluorescence, in renal disease

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Abstract

Introduction

Advanced glycation end-products (AGEs) accumulate during renal failure and dialysis. Kidney transplantation is thought to reverse this accumulation by restoring renal function.

Methods

Using a non-invasive and validated autofluorescence reader, we evaluated AGE levels (skin-AF) in 285 transplant recipients (52 [41-60] years), 32 dialysis patients (56 [43-65] years), and 231 normal control subjects (51 [40-65] years). Measurements in transplant recipients were performed 73 [32-143] months after transplantation. Dialysis patients were on dialysis therapy for 42 [17-107] months.

Results

Skin-AF was significantly increased in dialysis patients compared with normal control subjects (2.8 vs 2.0 a.u., $P<0.0001$). Although skin-AF levels were significantly decreased in transplant recipients compared with dialysis patients (2.5 vs 2.8 a.u., $P<0.0001$), skin-AF in transplant recipients was higher than controls (2.5 vs 2.0 a.u., $P<0.0001$). In transplant recipients skin-AF correlated positively with the duration of dialysis prior to transplantation (r=0.21, $P<0.0001$), and negatively with creatinine clearance (r=-0.34, $P<0.0001$). No correlation was found between time after transplantation and skin-AF in transplant recipients (r=-0.10, $P=0.10$). Skin-AF in dialysis patients was positively correlated with duration of dialysis (r=0.36, $P=0.042$).

Conclusions

Our results, like that of others, suggest that kidney transplantation not fully corrects increased AGE levels found in dialysis patients. The increased AGE levels in kidney transplant recipients cannot be explained by the differences in renal function alone. The availability of a simple, noninvasive method (AGE-Reader) to measure AGE-accumulation may be used to monitor AGE-accumulation in a clinical setting as well as in a study setting.

Introduction

Advanced glycation end-products (AGEs) compose a group of compounds formerly thought to be produced only by reactions between sugar adducts and proteins.[1] Nowadays, it is known, that not only sugar adducts, but also lipid adducts can react with proteins. In short, radicalized sugar and lipid adducts, also known as reactive carbonyl compounds react with proteins to form both AGEs and advanced lipoxidation end-products.[2,3] AGE-accumulation has been associated with aging,[4] renal function impairment,[5] hypertension,[6,7] smoking,[8] and the presence of diabetes.[9] AGEs accumulate both during the period of gradual renal function loss in the period prior to dialysis, and during dialysis treatment.[5] It is thought that increased AGE-accumulation in renal failure is mainly a consequence of decreased renal clearance of AGE compounds. Thus, kidney transplantation is though to lower AGE-accumulation by correcting renal function.

Although several methods to determine AGE-accumulation have been described, no commercial assay or tool is available yet. Traditionally, AGEs are determined using their characteristic autofluorescence properties.[10] Currently, gas chromatography mass spectrometry (GC-MS) is considered to be the most accurate technique to determine AGE-levels.[11] Other techniques include high performance liquid chromatography (HPLC),[12] enzyme-linked immunosorbent assay (ELISA),[13] and immunohistochemical techniques.[14] Recently, we developed a technique based upon the auotofluorescence properties of AGEs to estimate AGE-accumulation rapidly and noninvasively in vivo.[15] In the current study, we examine the suitability of this technique to measure AGE-accumulation in a large group of transplant recipients and compare these results with our results from control patients and dialysis patients.

Methods

Patients and study design

The study protocol was approved by the Institutional Review Board of Groningen University Medical Center (METc 01/039). We analysed AGE-accumulation in kidney transplant recipients, hemodialysis patients and normal control subjects. Kidney transplant recipients were all transplanted at Groningen University Medical Center, and survived the first year after transplantation with a functioning allograft, as described previously.[16] Dialysis patients were all undergoing hemodialysis therapy at Dialysis Center Groningen. Control subjects were admitted to hospital for different surgical interventions, unrelated to cardiovascular, renal, and/or inflammatory disorders. Diabetic patients and patients with history of renal disease were excluded from the control group. Non-Caucasian patients were excluded from analysis as the AGE-reader used for the determination of AGE-accumulation has not yet been validated in populations with increased skin pigmentation.

AGE-accumulation measured as skin-autofluorescence

AGE-accumulation was assessed by measuring skin-autofluorescence (skin-AF) using

the AGE-Reader (patent PCT/NL99/00607; Diagnoptics BV, Groningen, The Netherlands). In short, the AGE-reader illuminates a skin surface of approximately 1 cm^2, guarded against surrounding light, with an excitation light source between 300-420 nm (peak excitation ~ 370 nm). Light from the skin is measured with a spectrometer (AVS-USB2000, Avantes Inc., Eerbeek, The Netherlands) in the 300-600 nm range, using 200 µm glass fiber (Avantes UV/VIS, 200-750 nm, Avantes Inc., Eerbeek, The Netherlands). As a measure of skin-AF the ratio between emission and excitation was calculated in arbitrary units (a.u.) by dividing the area under the curve between 420-600 nm by the area under the curve between 300-420 nm, and multiplying by 100.[17] All measurements were performed at room temperature in a dark environment. Skin-AF was measured at the volar side of the lower arm, and the posterior side of the lower leg at approximately 10-15 cm below the elbow fold, respectively the hollow of the knee. Skin-AF measurements in an individual patient consisted of 75 measurements with an integration time of 75 ms. Care was taken to perform the measurement at normal skin site, i.e. without visible vessels, scars, lichenification, or other skin abnormalities. The AGE-reader has been validated previously in diabetic and control subjects against tissue AGE content of skin biopsies.[15] Intraobserver variation of repeated skin-AF measurements on one day was 6% in both diabetic and control subjects. Intra-individual seasonal variance among control subjects and diabetic patients was 6%.

Demographics, anthropometrics, and laboratory assessments

Smoking status was obtained from a self-report questionnaire. Drug-use was obtained from the medical record. During the visit to the outpatient clinic blood pressure was measured, and patients height and weight were assessed. Furthermore, blood and urine samples were collected. Using standard laboratory techniques blood was analysed for glucose and creatinine (only in kidney transplant recipients). Creatinine clearance in kidney transplant recipients was calculated from 24 hour urine by the UxV/P formula. Smoking was defined as current use of cigarettes. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Obesity was defined as BMI of 30 kg/m^2 or more.[18] According to the 2003 guidelines of the European Society of Hypertension, patients with a systolic blood pressure over 140 mmHg, a diastolic blood pressure over 90 mmHg, or patients using anti-hypertensive drugs, were considered to be hypertensive.[19] Diabetes mellitus was classified according to the criteria of the expert committee on the diagnosis and classification of diabetes mellitus as a fasting glucose higher than 6.9 mmol/l or the use of antidiabetic medication or insulin.[20]

Statistical analysis

Analyses were performed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Parametric parameters are expressed as mean $\pm$ SD, whereas non-parametric parameters are expressed as median [25%-75% IQR]. Nominal parameters are expressed as n(%). Differences between continuous variables were analysed using the independent samples T-test (Student's T-test). Differences between nominal variables were analysed using Pearson's Chi-square test. The Spearman's Rho test was used to correlate continuous parameters. A two-sided *P*-value of 0.05 or less was considered to indicate statistical significance.

Results

A total of 285 transplant recipients (52 [41-60] years), 32 dialysis patients (56 [43-65] years), and 231 normal control subjects (51 [40-65] years) were analysed. Transplant recipients were studied 73 [32-143] months post transplantation. Transplant recipients had previously been on dialysis for 26 [12-48] months. Dialysis patients were on dialysis for 42 [17-107] months. Creatinine clearance in transplant recipients was 62 ± 22 ml/min. Control subjects had no history of renal disease. Table 1 summarizes characteristics of patients and controls. Transplant recipients were significantly younger than dialysis patients (52 vs 56 years, P=0.016). Transplant recipients had significantly more hypertension than controls (97% vs 55%, P<0.0001) and dialysis patients (97% vs 63%, P<0.0001). Furthermore, transplant recipients smoked significantly less frequently than control subjects (20% vs 30%, P=0.005).

Table 1. Characteristics of transplant patients, dialysis patients and control subjects

Variables	Controls (n=231)	Dialysis patients (n=32)	Transplant patients (n=285)
Sex (Male)	139 (60)	18 (56)	163 (57)
Age	51 [40-65]	56 [43-65]	52 [41-60]
Diabetes (yes)	0 (0.0)	3 (9)	50 (18)
Hypertension (yes)	126 (55)	20 (63)	276 (97)
Smoking (yes)	70 (30)	9 (28)*	56 (20)
Obesity (yes)	43 (19)	4 (13)	37 (13)
Fluorescence lower arm (a.u.)	2.0 [1.7-2.4]	2.8 [2.5-3.8]	2.5 [2.1-2.9]

*Continuous parameters are expressed as median [25%-75% IQR]; nominal parameters are expressed as n(%); Annotations: * n = 28.*

Skin-AF was significantly increased in dialysis patients compared with controls (2.8 vs 2.0 a.u., P<0.0001). Although skin-AF levels were significantly decreased in transplant recipients when compared to dialysis patients (2.5 vs 2.8 a.u., P<0.0001), skin-AF in transplant recipients remained above values in controls (2.5 vs 2.0 a.u., P<0.0001). These differences are further illustrated by figure 1 in which skin-AF is depicted for transplant recipients, dialysis patients, and control subjects divided over 3 age groups in an attempt to match for age differences. Figures 2a-c illustrate skin-AF levels in patients without hypertension (a), without smoking (b) and without diabetes (c). In the absence of these known factors associated with AGE-accumulation, skin-AF in transplant recipients remained well above normal control subjects.

In transplant recipients, dialysis patients, and controls subjects skin-AF correlated positively with age (r=0.45, P<0.0001; r=0.42, P=0.018; r=0.63, P=0.018; respectively). In transplant recipients skin-AF correlated positively with the duration of dialysis prior to transplantation (r=0.21, P<0.0001), and negatively with creatinine clearance (r=-0.34, P<0.0001). No correlation was found between time after transplantation and skin-AF in transplant recipients (r=-0.10, P=0.10). Skin-AF in dialysis patients was positively correlated with duration of dialysis (r=0.36, P=0.042).

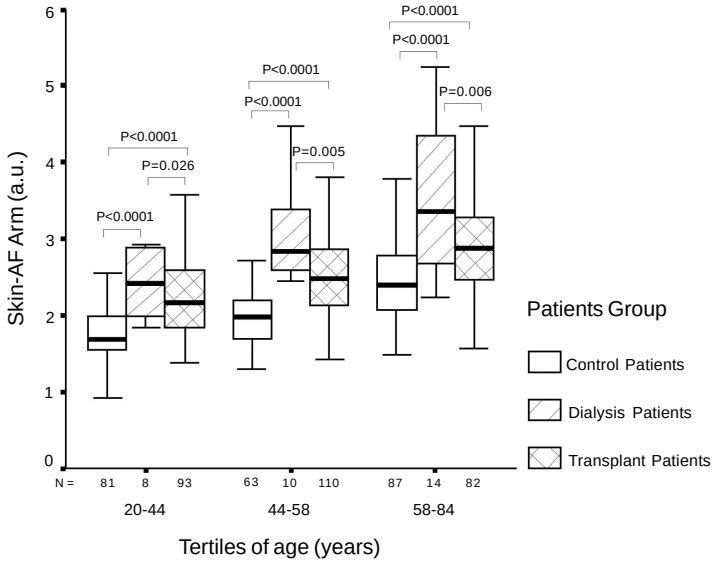

Figure 1. Skin-AF of transplant patients, dialysis patients and normal control subjects divided in tertiles of age.
P-values are calculated using independent samples T-test (student T-test).

Discussion

The results of this study show that AGE-accumulation as measured with the AGE-reader is increased in dialysis patients when compared with normal control subjects. Although AGE levels in kidney transplant recipients were significantly lower compared with dialysis patients, AGE levels remained well above normal control subjects. Our results on autofluorescence are in line with the results of others, that studied blood or tissue AGE levels. Recently, we summarized all studies available examining the influence of kidney transplantation on AGE-accumulation in blood and tissue.[21] We concluded, that although AGE levels were generally lower in transplant recipients compared with dialysis patients, levels remained well above normal control levels.

Renal function is known to be a very important determinant of AGE-accumulation.[5,22,23] It is most likely that the differences in fluorescence between control subjects, dialysis patients, and transplant recipients are largely explained by the existing differences in renal function. As we know, transplant recipients do not have fully normalized renal functions. This is more likely as both in transplant and in dialysis patients the duration of the episode without renal function (duration of dialysis) was related to the severity of AGE-accumulation. On the other hand, the duration of (partly) recovered renal function post transplant was not associated with AGE-accumulation, which suggests that other factors than renal function account for the increased AGE-accumulation after transplantation as well. In our dialysis and transplant patients, differences in fluorescence remain evident in those who did not smoke, and who did not have diabetes,

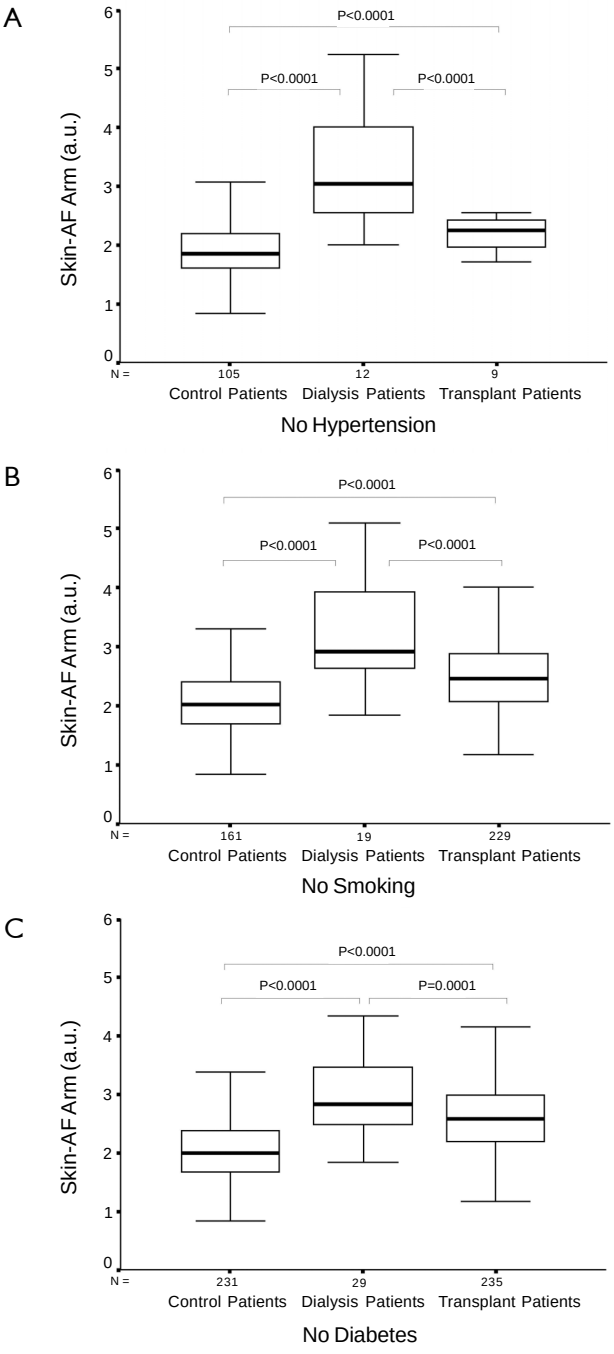

Figure 2a-c. Skin-AF of transplant patients, dialysis patients and control subjects in subgroups without hypertension (a), without smoking (b) and without diabetes (c).
P-values are calculated using independent samples T-test (student T-test).

or hypertension. Another factor may be the fact that the AGE-reader measures a pool of relatively irreversible AGEs. Restoration of renal function may therefore not completely correct AGE-accumulation. Another reason may be *de novo* creation of AGEs after transplantation. This may be the consequence of enhanced oxidative stress due to immunosuppressive medications such as cyclosporine[24] or due to changes in dietary intake of AGEs after transplantation.[8,25]

The AGE-reader was developed based upon the principle of the fluorescent properties of several (but not all) AGEs. Collagen linked fluorescence (CLF) has long been used as a single standard for measuring AGE-accumulation.[10] One limitation of the AGE-reader is that not all AGEs exhibit fluorescent properties. Indeed, fluorescence is a group reactivity, which fails to provide quantitative information on concentrations of individual compounds. Another limitation of the AGE-reader is that we cannot exclude the interference of other fluorophores in our measurement. Changes in skin fluorescence may also occur as a consequence of light absorption by chromophores such as melanin and hemoglobin.[26] Dialysis therapy may also have influenced fluorescence in our dialysis patients.[27] The AGE-reader was designed to measure tissue AGE-accumulation, but we cannot exclude that the AGE-reader measures both collagen linked fluorescence as well as fluorescence of fluids from the interstitial, cellular and vascular compartments. Thus differences in fluorescence between individual dialysis patients may partially be influenced by differences in dialysis quality.

In conclusion, our results, like that of others, suggest that kidney transplantation not fully corrects increased AGE levels found in dialysis patients. The increased AGE levels in kidney transplant recipients cannot be explained by the differences in renal function alone. The availability of a simple, noninvasive method (AGE-Reader) to measure AGE-accumulation may be used to monitor AGE-accumulation in a clinical setting as well as in a study setting.

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Chapter 7

Risk factors for chronic transplant dysfunction and cardiovascular disease are related to accumulation of advanced glycation end-products in renal transplant recipients

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Abstract

Introduction

Accumulation of advanced glycation end-products (AGEs) has been implicated in the pathogenesis of chronic transplant dysfunction and cardiovascular disease in renal transplant recipients. We aimed to investigate which factors are associated with tissue AGE-accumulation in renal transplant recipients.

Methods

AGE-accumulation (skin-AF) was assessed using a validated skin-autofluorescence reader (AGE-reader) in 285 consecutive renal transplant recipients (57% male, aged 50±12 years) visiting the outpatient clinic at a median (interquartile range) time of 73 (32–143) months after transplantation. Furthermore, various transplant- and recipient-related factors of interest were collected.

Results

Average skin-AF of lower arm and leg was 2.7±0.8 a.u. Skin-AF was positively determined by recipient age, systolic blood pressure, smoking, high-sensitivity C-reactive protein, duration of pre-transplant dialysis, and negatively by plasma vitamin C levels, creatinine clearance at baseline, and change in creatinine clearance since one year after transplantation in multivariable linear regression analysis. Together, these factors explained 41% of the variance of skin-AF.

Conclusions

Skin-AF was associated with several risk factors for cardiovascular disease and chronic renal transplant dysfunction. These results are in line with the hypothesis that AGEs play a role in the pathogenesis of these conditions in renal transplant recipients. Prospective studies are required to investigate whether the AGE-reader can be used as a simple, non-invasive tool to identify and monitor patients at risk for chronic renal transplant dysfunction and cardiovascular disease.

Introduction

Transplantation is currently the best renal replacement therapy for patients with end-stage renal disease. Graft loss due to cardiovascular mortality and chronic transplant dysfunction is a major concern in renal transplant medicine. Due to the introduction of new immunosuppressive medication short-term renal allograft survival has improved substantially. These improvements led to expectations of improved long-term survival rates. However, long-term survival rates still strongly lag behind.[1] One of the challenges in transplant research is to obtain insight into the factors associated with long-term allograft survival.

In transplant recipients, death rates from cardiovascular disease exceed those of the general population.[2] Most likely, this is the consequence of the high prevalence of cardiovascular risk factors in transplant recipients. The notion is emerging that the development of chronic renal transplant dysfunction constitutes, at least to a certain extent, a manifestation of cardiovascular disease.[3,4] This is supported by the fact that there is a great overlap between risk factors for cardiovascular disease and risk factors for chronic renal transplant dysfunction.[3,4] The latter include recipient age, impaired renal function, hypertension, the presence of diabetes, proteinuria, hyperlipidaemia, obesity, transplant ischaemia and use of calcineurin inhibitors.[3] Interestingly, these same risk factors also overlap factors associated with accumulation of advanced glycation end-products (AGEs). This led to the hypothesis that AGEs are involved in the development of cardiovascular disease and chronic renal transplant dysfunction after transplantation.[3,4]

Advanced glycation end-products (AGEs) originate from reactions between sugar and lipid adducts with proteins.[5] AGE-accumulation has been shown to increase with aging, renal function impairment and presence of diabetes in non-transplant populations. With respect to renal transplant recipients, AGE-accumulation might be further influenced by transplantation techniques, donor characteristics, human leucocyte antigen (HLA) mismatching, use of immunosuppressive medication and deteriorating renal function. We aimed to investigate the factors associated with tissue AGE-accumulation in renal transplant recipients.

Methods

Patients and study design

The study protocol was approved by the Institutional Review Board of the University Medical Center Groningen (METc 01/039). All renal transplant recipients transplanted at the University Medical Center Groningen were monitored after transplantation in the outpatient clinic in accordance with American Transplantation Guidelines,[2] i.e. ranging from twice a week just after discharge from hospital to twice a year long-term after transplantation. All adult patients (aged ≥18 years) who survived the first year after transplantation with a functioning allograft were eligible to participate (1 year post-transplant was considered baseline). From December 2001 to March 2003, we

invited 471 consecutive renal transplant recipients to participate in this study at their next visit to the outpatient clinic (index date). A total of 324 (69%) patients signed written informed consent. From this group, 12 patients were excluded from the analysis because of non-Caucasian ethnicity as the AGE-reader (AFR) used in this study to measure AGE-accumulation has not yet been validated in populations with increased skin pigmentation, and 27 patients because of missing data, leaving a total of 285 patient for analyses. All measurements were performed after an 8 h overnight fast.

AGE-accumulation measured as skin-autofluorescence

The AGE-accumulation was assessed by measuring skin-AF using a validated AGE-reader (AFR; patent PCT/NL99/00607; DiagnOptics BV, Groningen, The Netherlands) as described previously.[6] In short, the AGE-reader illuminates a skin surface of approximately 1 cm^2, guarded against surrounding light, with an excitation light source between 300 and 420nm (peak excitation~370 nm). Light from the skin is measured with a spectrometer (AVS-USB2000, Avantes Inc., Eerbeek, The Netherlands) in the 300–600 nm range, using 200 µm glass fibre (Avantes UV/VIS, 200–750 nm, Avantes Inc., Eerbeek, The Netherlands). As a measure of auto-fluorescence (skin-AF) the ratio between emission and excitation was calculated in arbitrary units (a.u.) by dividing the area under the curve between 420 and 600nm by the area under the curve between 300 and 420 nm, and multiplying by 100.[7] All measurements were performed at room temperature in a dark environment. Skin-AF was measured at the volar side of the lower arm, and the posterior side of the lower leg at approximately 10–15 cm below the elbow fold and the hollow of the knee, respectively. Skin-AF measurements in an individual patient consisted of 75 measurements with an integration time of 75 ms. Care was taken to perform the measurement at normal skin site, i.e. without visible vessels, scars, lichenification or other skin abnormalities. Intra-observer variation of repeated skin-AF measurements on one day was 6%. For data analysis we calculated the average skin-AF of arm and leg.

Recipient and transplant characteristics

Relevant recipient and transplant characteristics were partially extracted from the Groningen Renal Transplant Database. This database holds information of all renal transplantations that have been performed at our centre since 1968. Extracted were recipient and donor age, gender, primary renal disease, duration of pre-transplant dialysis, date of transplantation, ischaemia time, number of HLA mismatches, acute rejection treatment and 24 h urinary creatinine clearance at one year after transplantation. Current medication was extracted from medical records. History of cardiovascular disease and smoking status were obtained from a self-report questionnaire. Smoking was defined as current use of cigarettes. Patients were grouped as having experienced an episode of rejection, when drugs were used to treat rejection. History of cardio-vascular disease was based on patient self-report of myocardial infarction, angina pectoris, cerebrovascular accident, transient ischaemic attack or intermittent claudication in the medical history of the patient. Standard immunosuppression consisted of the following: from 1968 until 1989 prednisolone and azathioprine; from January 1989 until February

1993 ciclosporin standard formulation (Sandimmune, Novartis) combined with prednisolone; from March 1993 until May 1996 ciclosporin microemulsion (Neoral, Novartis Pharma b.v., Arnhem, The Netherlands) and prednisolone; from May 1997 to date mycophenolate mofetil (Cellcept, Roche b.v., Woerden, The Netherlands) was added.

Clinical measurements

During the visit to the outpatient clinic, blood pressure was measured using an automated oscillometric blood pressure device (Omron M4, Omron Europe b.v., The Netherlands) as the average of three consecutive measurements with 1 min intervals after a 6 min rest in the supine position. Height and weight were assessed as well. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Obesity was defined as BMI of 30 kg/m^2 or higher, according to the guidelines of the World Health Organization.[8] According to the 2003 guidelines of the European Society of Hypertension, patients with a systolic blood pressure 140 mmHg, a diastolic blood pressure 90 mmHg, or patients using anti-hypertensive drugs, were considered to be hypertensive.[9]

Laboratory assessments

Blood was drawn at the outpatient clinic and 24 h urine samples were collected. Using standard laboratory techniques urine was assessed for concentrations of protein and creatinine, and blood was analysed for concentrations of creatinine, glucose and total cholesterol. Vitamin C and E were determined by HPLC (Knauer K-1001, Wissenschaftliche Geratebau, Berlin, Germany; Waters 717 PLUS, Milford, MA, USA; Shimadzu RF 551, Shimadzu Scientific Instruments Inc., Maryland, Columbia, USA). HbA1c was determined by HPLC as well (VARIANTTM HbA1c Program with Bio-Rad CARIANT Hb Testing System, Bio-Rad, Hercules, CA, USA). Serum was assessed with a high-sensitivity (hs) CRP ELISA assay. Both intra- and inter-assay variation coefficients were 5%. Creatinine clearance was calculated from 24 h urine by the UxV/P formula. Delta creatinine clearance was calculated by subtracting creatinine clearance at index date from creatinine clearance at baseline. Hypercholesterolaemia was defined as a total cholesterol higher than 6.2 mmol/l or use of lipid lowering drugs (statins), according to the National Cholesterol Education Program (NCEP) criteria.[10] Diabetes mellitus was classified according to the criteria of the expert committee on the diagnosis and classification of diabetes mellitus as a fasting glucose higher than 6.9 mmol/l or the use of antidiabetic medication or insulin.[11]

Statistical analyses

Analyses were performed using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA). Parametric variables are expressed as mean±SD, whereas non-parametric variables are expressed as median (interquartile range). Nominal variables are expressed as n (%). To gain insight into which risk factors are associated with AGE-accumulation, we first performed univariable analyses for trend over quartiles of skin-AF. *P*-value for trend was determined by linear regression for continuous variables, and by chi-

square and Jonckheere–Terpstra tests for nominal and ordinal variables, respectively. Second, univariable linear regression analyses were performed for factors that showed at least a trend ($P \leq 0.20$) with skin-AF in trend analyses (model 1). Adjustments were consecutively performed for age (model 2) and renal function parameters (model 3). Third, to analyse which of the factors were independently associated with skin-AF, a multivariable linear regression analysis was performed with skin-AF as dependent variable. Next to age and renal function parameters, co-variables with a *P*-value ≤0.20 in model 3 were included in the analysis. Variables which have not retained significance in this multivariable analysis were subsequently removed from the model (socalled backward selection). This method is most suitable for cross-sectional data. To test whether the model is appropriate and whether the assumptions for linear regression are met, the model has been tested for overall regression, collinearity, interaction terms and lack-of-fit with ANOVA. Residuals were tested for normality of distribution. A *P*-value ≤0.05 was considered to indicate statistical significance.

RESULTS

Results are presented for a total of 285 transplant recipients (163 male, 122 female). Mean age of transplant recipients was 50±12 years. Index date was 73 (32–143) months after transplantation. At index date 96.8% of patients had hypertension, 15.8% had diabetes mellitus, 13.0% were obese and 66.0% had hypercholesterolaemia. Creatinine clearance at index date was 62±22 ml/min. Skin-AF of lower arm and leg at index date were 2.6±0.7 a.u. and 2.9±1.0 a.u., respectively, with an average of 2.7±0.8 a.u.

Tables 1 and 2 show recipient and transplant characteristics grouped according to quartiles of skin-AF. Significant associations with skin-AF were present for recipient sex, recipient age, systolic blood pressure, HbA1c, plasma vitamin C levels, hs-CRP, smoking, history of cardiovascular disease, donor age, duration of pre-transplant dialysis, creatinine clearance at baseline, creatinine clearance at index date and delta creatinine clearance. No effect of immunosuppressive treatment and the use of ACE inhibition (AII receptor antagonists or ACE inhibitors) was found.

Using univariable linear regression analysis we calculated standardised regression coefficients (β) and *P*-values for the variables that at least showed a tendency ($P \leq 0.20$) to be associated with skin-AF in trend analyses (model 1). Adjustments were consecutively performed for age (model 2), and renal function parameters (model 3). The effect of adjustments can be judged by comparing standardised regression coefficients and *P*-values of an association before and after adjustment (table 3). Adjustments for age and renal function parameters did not substantially affect the association between plasma vitamin C and skin-AF. The association between smoking and skin-AF strengthened after adjustment for age. The associations of BMI, total time of ischaemia, HbA1c and history of cardiovascular disease with skin-AF were less strong after adjustment for age. The associations of durations of dialysis prior to transplantation, and donor age became less strong after adjustment for renal function parameters. The associations of recipient sex, systolic blood pressure and hs-CRP were (partially) dependent on both age and renal function parameters.

Table 1. Recipient characteristics grouped according to quartiles of skin-AF

Recipient characteristics	Quartiles of skin-AF (a.u.)				*P* for trend
	1.2–2.2 (*n*=71)	2.2–2.6 (*n*=71)	2.6–3.1 (*n*=72)	3.1–5.2 (*n*=71)	
Recipient demographics					
Recipient sex (male)	44 (62.0)	48 (67.6)	38 (52.8)	33 (46.5)	0.02
Recipient age (years)	44 ± 11	47 ± 11	52 ± 12	58 ± 10	< 0.001
Primary renal disease, n (%)					
Primary glomerulopathy	26 (36.6)	20 (28.2)	21 (29.2)	15 (21.1)	> 0.2
Tubulointerstitial/pyelonephritis	11 (15.5)	13 (18.3)	9 (12.5)	11 (15.5)	
Cystic renal disease	10 (14.1)	9 (12.7)	8 (11.1)	13 (18.3)	
Vasculitis/autoimmune	6 (8.5)	7 (9.9)	4 (5.6)	3 (4.2)	
Other	18 (25.3)	22 (30.9)	30 (41.6)	29 (40.9)	
Clinical measurements					
Systolic blood pressure (mmHg)	144 ± 19	147 ± 18	153 ± 21	165 ± 26	< 0.001
Diastolic blood pressure (mmHg)	89 ± 10	89 ± 9	88±11	92 ± 10	0.11
Body mass index (kg/m2)	25.1 ± 3	7 25.5 ± 3.7	25.7 ± 4.9	26.5 ± 4.6	0.06
Laboratory assessments					
HbA1c (%)	6.2 ± 0.9	6.3 ± 1.1	6.5 ± 1.0	6.8 ± 1.2	< 0.001
Fasting glucose (mmol/l)	4.7 ± 1.0	5.0 ± 1.3	5.0 ± 1.3	4.9 ± 1.1	0.20
Total cholesterol (mmol/l)	5.5 ± 0.9	5.3 ± 1.0	5.6 ± 1.0	5.7 ± 1.1	0.14
Serum albumin (g/l)	41 ± 2.8	41 ± 3.8	40 ± 4.2	40 ± 3.5	0.07
Plasma vitamin C (mmol/l)	50 ± 18	46 ± 20	48 ± 21	37 ± 21	0.001
Plasma vitamin E (mmol/l)	35 ± 10	38 ± 12	37 ± 11	37 ± 12	> 0.2
hs-CRP (mg/ml)	1.4 [0.6-2.8]	1.2 [0.7-3.5]	1.7 [0.7-4.1]	3.2 [1.2-7.3]	0.01
Proteinuria (g/24 h)	0.2 [0.0-0.4]	0.2 [0.1-0.5]	0.2 [0.0-0.5]	0.3 [0.0-0.7]	> 0.2
Questionnaire results					
Smoking, n (%)	9 (12.7)	12 (16.9)	15 (20.8)	20 (28.2)	0.02
History of CVD, n (%)	6 (8.5)	4 (5.6)	10 (13.9)	12 (16.9)	0.05
Drug-use of interest					
Use of prednisolon, n (%)	71 (100)	71 (100)	72 (100)	71 (100)	> 0.2
Dose (mg/day)	10 [7.5-10]	10 [8.8-10]	10 [7.5-10]	10 [8.8-10]	> 0.2
Use of calcineurin inhibitors					
Ciclosporine, n (%)	44 (62.0)	42 (59.2)	47 (65.3)	47 (66.2)	> 0.2
Trough-level (g/l)	108 ± 44	111 ± 47	107 ± 45	117 ± 50	> 0.2
Tacrolimus, n (%)	10 (14.1)	13 (18.3)	13 (18.1)	9 (12.7)	> 0.2
Trough-level (g/l)	9 ± 2	9 ± 4	8 ± 3	10 ± 8	> 0.2
Use of proliferation inhibitors					
Azathioprine, n (%)	25 (35.2)	26 (36.6)	23 (31.9)	22 (31.0)	> 0.2
Mycophenolate mofetil, n (%)	27 (38.0)	31 (43.7)	27 (37.5)	29 (40.8)	> 0.2
Use of ACE inhibition, n (%)					
All receptor antagonist, n (%)	3 (4.2)	7 (9.9)	5 (6.9)	5 (7.0)	> 0.2
ACE inhibitors, n (%)	21 (29.6)	20 (28.2)	24 (33.3)	16 (22.5)	> 0.2

Parametric parameters are expressed as mean±SD; non-parametric parameters are expressed as median (25–75% IQR); ordinal parameters are expressed as n (%). CVD, cardiovascular disease.

Using multivariable linear regression analysis we determined which factors were independently associated with skin-AF. Variables that showed significant association—or at least a tendency to be significant ($P \leq 0.20$)—with skin-AF after adjustments for age and renal function parameters (model 3) were entered in to our model. A summary

Table 2. Transplant characteristics grouped according to quartiles of skin-AF

Transplant characteristics	Quartiles of skin-AF (a.u.)				*P* for trend
	1.2–2.2 (*n*=71)	2.2–2.6 (*n*=71)	2.6–3.1 (*n*=72)	3.1–5.2 (*n*=71)	
Donor demographics					
Donor sex (male)	42 (59.2)	36 (50.7)	35 (48.6)	40 (56.3)	> 0.2
Donor age (years)	36 ± 15	37 ± 16	36 ± 15	42 ± 15	0.04
Duration of pre-transplant dialysis (months)	28 ± 35	31 ± 26	40 ± 53	42 ± 32	0.01
Type of transplantation, n (%)					
Post-mortem donor	60 (84.5)	57 (80.3)	56 (77.8)	60 (84.5)	> 0.2
Living donor	9 (12.7)	13 (18.3)	13 (18.0)	8 (11.3)	
Renal and pancreas	2 (2.8)	1 (1.4)	3 (4.2)	3 (4.2)	
HLA-AB mismatches, n (%)					
0	20 (28.2)	14 (19.7)	27 (37.5)	24 (33.8)	> 0.2
1–2	44 (61.9)	52 (73.3)	32 (44.4)	39 (54.9)	
3–4	7 (9.9)	5 (7.0)	13 (18.1)	8 (11.3)	
HLA-DR mismatches, n (%)					
0	48 (67.6)	36 (50.7)	50 (69.4)	44 (62.0)	> 0.2
1–2	23 (32.4)	35 (49.3)	22 (30.6)	27 (38.0)	
Total time of ischaemia (h)	23 ± 11	21 ± 11	23 ± 16	26 ± 17	0.18
Transplant follow-up					
Time elapsed since baseline (months)	62 [31-134]	60 [25-131]	71 [19-124]	58 [12-137]	> 0.2
Acute rejection, n (%)	38 (53.5)	33 (46.5)	37 (51.4)	27 (38.0)	0.12
Creatinine clearance at baseline (ml/min)	69 ± 19	69 ± 20	65 ± 19	58 ± 18	< 0.001
Creatinine clearance at index (ml/min)	71 ± 19	67 ± 21	60 ± 24	52 ± 20	< 0.001
Delta creatinine clearance (ml/min)	2 ± 17	-2 ± 17	-6 ± 21	-6 ± 18	0.01

Parametric parameters are expressed as mean±SD; non-parametric parameters are expressed as median (25–75% IQR); ordinal parameters are expressed as n (%).

of the multivariable regression model is given in table 4. In our model, 41% of the variation of skin-AF was positively determined by recipient age, systolic blood pressure, smoking, hs-CRP, duration of pre-transplant dialysis, and negatively by plasma vitamin C levels, creatinine clearance at baseline and change in creatinine clearance since 1 year after transplantation. Although no association was observed for time elapsed beyond 1 year after transplantation in univariable analysis, we evaluated its influence in the multivariable model. However, time elapsed since baseline did not significantly contribute to the model. Furthermore, as diabetes mellitus is a potent trigger to AGE formation we evaluated its influence on our results. However, exclusion of patients with diabetes mellitus did not materially affect the results of our analyses.

Table 3. Regression analysis with recipient- and transplant-related factors; influences of age and renal function on model relations

Determinants	Model 1		Model 2		Model 3	
	β	*P*	β	*P*	β	*P*
Recipient sex (female)	0.14	0.02	0.10	0.08	0.05	> 0.2
Recipient age (years)	0.44	< 0.001	-	-	-	–
Duration of pre-transplant dialysis (months)	0.15	0.01	0.14	0.007	0.10	0.06
Systolic blood pressure (mmHg)	0.32	< 0.001	0.20	0.001	0.16	0.003
Diastolic blood pressure (mmHg)	0.06	> 0.2	0.08	0.13	0.06	> 0.2
Body mass index (kg/m2)	0.10	0.11	0.01	> 0.2	0.08	0.14
HbA1c (%)	0.24	< 0.001	0.12	0.04	0.10	0.05
Fasting glucose (mmol/l)	0.07	> 0.2	0.00	> 0.2	0.04	> 0.2
Total cholesterol (mmol/l)	0.06	> 0.2	0.04	> 0.2	0.02	> 0.2
Serum albumin (g/l)	- 0.14	> 0.2	- 0.09	> 0.2	- 0.06	> 0.2
Plasma vitamin C (mmol/l)	- 0.23	< 0.001	- 0.23	< 0.001	- 0.22	< 0.001
hs-CRP (µg/ml)	0.21	0.001	0.17	0.002	0.11	0.03
Smoking, n (%)	0.16	0.009	0.19	< 0.001	0.18	< 0.001
History of CVD, n (%)	0.12	0.04	0.06	> 0.2	0.04	> 0.2
Donor age (years)	0.19	0.001	0.16	0.002	0.08	0.14
Total time of ischaemia (h)	0.08	0.20	- 0.01	> 0.2	- 0.04	> 0.2
Acute rejection, n (%)	- 0.06	> 0.2	- 0.03	> 0.2	- 0.04	> 0.2
Creatinine clearance at baseline (ml/min)	- 0.27	< 0.001	- 0.21	< 0.001	-	-
Creatinine clearance at index (ml/min)	- 0.39	< 0.001	- 0.34	< 0.001	-	-
Delta creatinine clearance (ml/min)	- 0.18	0.003	- 0.18	0.001	-	-

β, standardized regression coefficients; CVD, cardiovascular disease.
Model 1 is the crude model; model 2 is corrected for age; model 3 is corrected for age and renal function parameters (baseline creatinine clearance, creatinine clearance at index and delta creatinine clearance).

Table 4. Determinants for skin-AF in a multivariable regression model

Determinants	Regression coefficients (R^2=0.41, adjusted R^2=0.39, *P*<0.001)			
	β	B	CI of B	*P*-value
Constant	-	1.411	0.726-2.096	< 0.001
Recipient age (years)	0.36	0.022	0.016-0.029	< 0.001
Systolic blood pressure (mmHg)	0.17	0.006	0.002-0.009	0.002
Smoking	0.13	0.25	0.068-0.438	0.008
hs-CRP (µg/ml)	0.12	0.007	0.001-0.013	0.02
Duration of pre-transplant dialysis (months)	0.11	0.002	0.000-0.004	0.03
Plasma vitamin C (mol/l)	- 0.15	- 0.005	- 0.009 to -0.002	0.003
Creatinine clearance at baseline (ml/min)	- 0.24	- 0.009	- 0.013 to -0.005	< 0.001
Delta creatinine clearance (ml/min)	- 0.24	- 0.010	- 0.014 to -0.005	< 0.001

β, standardized regression coefficients; B, unstandardized regression coefficient; CI, confidence interval.

Discussion

We found recipient age, systolic blood pressure, smoking, hs-CRP, duration of pre-transplant dialysis, plasma vitamin C levels, creatinine clearance at baseline and change in creatinine clearance since 1 year after transplantation to be independently associated with AGE-accumulation after renal transplantation. To the best of our knowledge no other investigators have systematically analysed determinants of AGE-accumulation in transplant recipients. We showed that AGE-accumulation is associated with multiple cardiovascular risk factors in transplant recipients. Furthermore, we showed that AGE-accumulation is related to transplant-specific factors. The latter include baseline renal function, decrease in renal function over time, donor age and duration of dialyses prior to transplantation.

The process of AGE-accumulation is time-dependent and influenced by AGE production on the one hand and AGE breakdown and clearance by the kidneys on the other.[3,5] As expected the most compelling factors associated with AGE-accumulation in our study were time-dependent as well. Strongly and independently associated with AGE-accumulation was age. This finding confirms earlier observations in renal transplant recipients.[12] Our results also indicate that renal function is an important determinant of AGE-accumulation. Renal function may be responsible for AGE-accumulation, both because of disturbed clearance of AGEs and intermediate products, and due to increased oxidative stress.[3] The fact that AGE-accumulation is strongly associated with baseline as well as index renal function suggests that the formation, accumulation, breakdown and clearance of tissue AGEs as measured by skin-AF in our study is a slow process. This idea is supported as well by the finding that duration of pre-transplant dialysis was independently associated with AGE-accumulation after transplantation. The relation between duration of pre-transplant dialysis and skin-AF was at least partially determined by renal function as can be concluded from the decreasing standardised regression coefficients after adjustment for renal function depicted in table 3. A similar pattern occurs for donor age implicating that a lower creatinine clearance intrinsic to older kidneys partially explains the relationship of donor age with AGE-accumulation.

The independent relation of smoking with AGE-accumulation is likely to be caused by reactive glycation adducts in cigarette smoke. Glycation adducts in cigarette tobacco are able to form cross-links with proteins.[13] Furthermore, it has been demonstrated, that smokers have significantly more serum AGEs than non-smokers.[13]

Oxidative stress and inflammation are believed to be involved in the pathogenesis of chronic renal transplant dysfunction, and are also intricately linked to AGE formation.[3,5] Although we found that vitamin C and hs-CRP were related independently to AGE formation, these markers do not provide conclusive information on oxidative stress and inflammation in our patients.

While HbA1c was associated with AGE-accumulation univariately, HbA1c was not independently associated with AGE-accumulation in our group. In diabetic patient groups, HbA1c is known to be independently associated with AGE-accumulation.[3,5] From table 3 it can be concluded that the relation between HbA1c and skin-AF in our

group was mainly determined by age. Probably, this is related to the small percentage of diabetics in our group (15.8% at index date).

The independent relationship between systolic blood pressure and AGE-accumulation has been reported previously.[14] In contrast with most of the factors discussed above we assume that this factor mainly represents a consequence of AGE-accumulation, rather than a cause. It suggests that AGEs might be involved in the development of vascular stiffness, resulting in hypertension. However, enhanced wall tension in blood vessels and cardiac tissue due to hypertension is thought to enhance oxidative stress and might thereby result in enhanced AGE-accumulation.

Immunosuppressive drugs (mainly ciclosporin) and the use of ACE inhibition have previously been associated with oxidative stress and AGE-accumulation. ACE inhibition with either ACE inhibitors or AII-receptor antagonists has been shown to decrease AGE-accumulation.[15,16] Ciclosporin has been reported to aggravate oxidative stress, possibly leading to enhanced AGE-accumulation.[17] We did not find a relationship between the use of ACE inhibition and/or immunosuppressive drugs and AGE-accumulation. Caution should be used in the interpretation of the lack of relation, because of the variability of duration of exposure of these drugs. The fact that tissue AGEs are thought to have a longer half-life than plasma AGEs may be another explanation for the lack of correlation found.

AGE-accumulation was determined using our newly developed and validated AGE-reader. This tool is based upon the principle of the fluorescent properties of several (but not all) AGEs. Collagen linked fluorescence has long been used as a single standard for measuring AGE-accumulation. One limitation of the AGE-reader is that not all AGEs exhibit fluorescent properties. Indeed, fluorescence is a group reactivity, which fails to provide quantitative information on concentrations of individual compounds. Another limitation of the AGE-reader is that we cannot exclude the interference of other fluorophores in our measurement. Changes in skin-AF may also occur as a consequence of light absorption by chromophores such as melanin and haemoglobin. Our findings are limited as well by the fact that it can not be concluded whether skin AGE levels are an independent risk factor for the development of cardiovascular disease as well as chronic transplant dysfunction in our group. On the one hand this is the consequence of the cross-sectional nature of our study. On the other hand this is due to the absence of a control group of the general population. A further limitation of our study is that we do not have preoperative AGE level data. This would have been of interest as many facets changed post-transplant, particularly renal function (thus affecting AGE clearance) and the institution of highly potent antirejection medications.

In conclusion, increased accumulation of AGEs measured as skin-AF in vivo is associated with several risk factors for chronic renal transplant dysfunction and cardiovascular disease. Some of these relations are suggestive for a causative role of AGEs in chronic transplant dysfunction and cardiovascular disease in renal transplant recipients. Prospective studies and/or future intervention studies with AGE-lowering therapy may allow to more definitely determine the relative role of AGE-accumulation in the development of chronic renal transplant dysfunction and cardiovascular disease after transplantation. The availability of a simple, non-invasive method to measure

AGE-accumulation in renal transplant recipients may be useful in identifying and monitoring patients at risk for AGE-accumulation.

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Chapter 8

Skin-autofluorescence is an independent predictor of graft loss in renal transplant recipients

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Abstract

Introduction

Skin-autofluorescence (skin-AF) non-invasively measures the tissue accumulation of advanced glycation end-products (AGEs). AGEs are nephrotoxic and potential effectors of cardiovascular mortality. We investigated, whether skin-AF predicted graft loss after kidney transplantation.

Methods

A total of 302 renal transplant recipients were enrolled at a median time of 6.1 [2.6-12.1] years after transplantation and were subsequently followed-up for first occurrence of graft loss (i.e. graft failure or all-cause mortality) for 5.2 [4.6-5.4] years. The association of baseline skin-AF with graft loss was investigated with univariable and multivariable Cox-regression and receiver-operator-characteristic (ROC) curve analyses.

Results

Baseline skin-AF was 2.7 ± 0.8 arbitrary units. Skin-AF predicted graft loss in a univariable Cox regression analysis (HR 2.40 [1.75-3.29], $P<0.001$) and in a multivariable model (HR 1.83 [1.22-2.75], $P=0.003$), adjusted for other identified risk-factors, including patient age, creatinine clearance, protein excretion, hsCRP, and HLA-DR mismatching. The area under the ROC curve for skin-AF as predictor of graft loss was significantly different from 0.5. Skin-AF was also a significant predictor of graft failure and mortality as separate end-points.

Conclusions

We conclude that skin-AF is an independent predictor of graft loss in kidney transplant recipients. Although skin-AF is not a direct measurement for AGEs, we feel that our results do support the hypothesis that accumulation of AGEs in renal transplant recipients contributes to the development of graft loss.

Introduction

End-stage renal disease (ESRD) is an important medical problem in the Western world, which is expected to increase in the future.[1] ESRD is preferably treated with kidney transplantation since this treatment significantly enhances the quality of life and survival of patients in comparison to dialysis treatments.[2,3] Although the short-term success of kidney transplantation has improved steadily in recent years with efficient treatment protecting from acute rejection,[4] the long-term success still needs improvement. Patients find themselves threatened by the enhanced risk for mortality, and sometimes even more by the risk of being re-admitted to dialysis. As many as 60% of patients transplanted with a cadaveric donor kidney develop graft failure within 10 years after transplantation. Age-adjusted rates of mortality are approximately 3-5 times higher in renal transplant recipients than in the general population.[5,6]

Both graft failure and patient mortality have been hypothesized to result at least partly from the pathogenic effects of oxidative stress and advanced glycation end-products (AGEs).[7,8] Basically, oxidative stress causes protein damage such as protein glycation, the products of which can be recognised by a number of cellular receptors.[9] Receptor activation then induces prolonged pro-inflammatory signaling, which might lead to vascular damage, and finally may result in graft failure and mortality.[9]

Skin-autofluorescence (skin-AF) measurement is a newly developed non-invasive technique which has been validated to measure the accumulation of AGEs.[10] We previously found skin-AF to predict mortality in ESRD patients on dialysis.[11] In this study we investigate whether skin-AF is an independent predictor of graft loss in kidney transplant recipients.

Methods

Patients and study design

The study protocol was approved by the Institutional Review Board of the University Medical Center Groningen (METC 01/039). All renal transplant recipients transplanted at the University Medical Center Groningen are monitored in accordance with the American Transplantation Guidelines[12] in the outpatient clinic. Between August 2001 and July 2003, all adult allograft recipients who survived the first year after transplantation with a functioning allograft were eligible to participate at their next visit to the outpatient clinic. The aim of our study was to investigate AGE-accumulation as a potential determinant of long-term transplant survival. In the first year after transplantation graft loss is frequently related to acute rejection, urological problems, and infections. To avoid confounding by such events, we only considered patients eligible for participation in the study who were one year after transplantation or beyond. A total of 606 out of 847 eligible renal transplant recipients signed written informed consent. Skin-AF was measured in a sub-population consisting of 309 consecutive patients because the AGE-reader measurement was not yet available at study initiation. From this sub-population 7 non-Caucasian patients were excluded, because the skin-

AF measurement has not yet been validated for measurements in patients with pigmented skin. The group that did not sign informed consent was comparable with the group that signed informed consent with respect to age, sex, time since transplantation, creatinine clearance, and proteinuria.[13] Furthermore, no significant differences existed in donor age, recipient age, donor sex, recipient sex, diabetes, baseline creatinine clearance, and urinary protein excretion between the 302 patients in which skin-AF was recorded and the 304 patients in which skin-AF was not recorded. All measurements, including blood sampling were performed after an 8-12h overnight fasting period for food and medication.

Follow-up

Patients were enrolled at a median time of 6.1[2.6-12.1] years after transplantation and were subsequently followed for first occurrence of graft loss for 5.2 [4.6-5.4] years. Graft loss was considered to have occurred if patients were re-admitted to dialysis, if they were re-transplanted or if they died. Up-to-date and complete information on patient status was ensured by our outpatient program, which operates in close collaboration with referral hospitals in our area.

AGE-accumulation measured as skin-autofluorescence

The AGE-accumulation was assessed by measuring skin-AF using a validated autofluorescence reader (AGE Reader™, DiagnOptics b.v., Groningen, The Netherlands) as it was described previously.[10] In short, the autofluorescence reader illuminates a skin surface of 1 cm^2, guarded against surrounding light, with an excitation light source between 300 and 430 nm (peak excitation ~370nm). Light from the skin is measured with a spectrometer (AVS-USB2000, Avantes Inc., Eerbeek, The Netherlands) in the 300-600 nm range, using a 200 μm glass fiber (UV/VIS 200-750 nm, Avantes Inc., Eerbeek, The Netherlands). Skin-AF measurements in an individual patient consisted of 75 measurements, each with an integration time of 75ms. Skin-AF was measured as the ratio between emission and excitation calculated in arbitrary units (AU) by dividing the intensity of the fluorescent light coming from the skin (measured as area under the curve of fluorescent wave lengths between 420 and 600 nm) by the intensity of the emitted light (measured as area under the curve of wave lengths between 300 and 420 nm) multiplied by one hundred. All measurements were performed at room temperature in a dark environment. Skin-AF was measured at the volar side of the lower arm at approximately 10-15 cm below the elbow fold and the hollow of the knee, respectively. The average of both measurements was calculated for further analyses. Care was taken to perform the measurements at normal skin site, i.e. without visible vessels, scars, lichenification or other skin abnormalities. Intra-observer variation of repeated autofluorescence measurements on one day was 6%.

Recipient and transplant characteristics

Relevant donor, recipient and transplant characteristics were extracted from the Groningen Renal Transplant Database. Extracted were age and sex from both donors and recipients, duration of pre-transplant dialysis, date of transplantation, transplantation type, ischemia time, HLA mismatches, renal function at baseline, and type of acute

rejection treatment. History of cardiovascular disease (CVD) and smoking status were obtained from a self-report questionnaire. Smoking was defined as current use of cigarettes. History of CVD was based on patient self-report of myocardial infarction, angina pectoris, cerebrovascular accident, transient ischaemic attack or intermittent claudication in the medical history of the patient. Current medication was extracted from medical record. Patients were defined as having experienced an episode of rejection, when drugs were used to treat rejection. Standard immunosuppression consisted of the following: from 1968 until 1989 prednisolone and azathioprine; from January 1989 until February 1993 cyclosporine standard formulation (Sandimmune, Novartis) combined with prednisolone; from March 1993 until May 1996 cyclosporine microemulsion (Neoral, Novartis Pharma b.v., Arnhem, the Netherlands) and prednisolone; and from May 1997 to date mycophenolate mofetil (Cellcept, Roche b.v., Woerden, The Netherlands).

Clinical measurements

During the visit to the outpatient clinic, blood pressure was measured using an automated oscillometric blood pressure device (Omron M4, Omron Europe b.v., the Netherlands) as the average of 3 consecutive measurements with 1-minute intervals after a 6 minutes rest in supine position. During this visit also height and weight were assessed and the body mass index (BMI) was calculated. According to the 2003 guidelines of the European Society of Hypertension, patients were considered to be hypertensive, if they had a systolic blood pressure over 140 mmHg, if they had a diastolic blood pressure over 90 mmHg, or if they used anti-hypertensive drugs.

Laboratory measurements

Blood was drawn at the outpatient clinic and 24-hour urine samples were collected. Urine was assessed for concentrations of protein and creatinine, and blood was analysed for concentrations of glucose, and total cholesterol using standard laboratory techniques. HbA1c was determined by HPLC (VARIANTTM HbA1c Program with Bio-Rad CARIANT Hb Testing System, Bio-Rad, Hercules, CA, USA). Serum CRP was assessed with a high-sensitivity CRP ELISA assay as described before.[14] Serum triglycerides were determined with the GPO-PAP method (MEGA AU 510, Merck Diagnostica Darmstadt, Germany). Creatinine clearance was determined from 24-hour urine samples. HDL-cholesterol was determined using the CHOD-PAP method (Technikon RA-1000, Bayer Diagnostics b.v., Mijdrecht, The Netherlands). LDL-colesterol was calculated using the Friedewald formula.[15] Hypercholesterolemia was defined as a total cholesterol higher than 6.2 mmol/l or use of lipid lowering drugs (statins), according to the National Cholesterol Education Program (NCEP) criteria.[16] Diabetes mellitus was classified according to the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus as a fasting glucose higher than 6.9 mmol/l or the use of anti-diabetic medication or insulin.[17]

Statistical analyses

Analyses were performed with SPSS version 14 (SPSS Inc., Chicago, IL, USA). Parametric variables are expressed as mean ± standard deviation, non-parametric

variables are given as median [25%-75% interquartile range] and nominal variables are given as n (%). Hazard ratios (HR) and areas under the ROC curves are displayed with 95% confidence intervals [95%CI]. For all analyses a *P*-value of 0.05 was considered to indicate statistical significance. All baseline variables were stratified for skin-AF tertiles and tested for difference over the respective tertiles. Tertile 1 was defined as skin-AF between 1.2-2.3 a.u., tertile 2 as skin-AF between 2.3-2.9 a.u., and tertile 3 as skin-AF between 2.9-5.2 a.u. Parametric variables were tested using one-way ANOVA, nominal variables using the chi-square test, and non-parametric variables using the Jonckheere-Terpstra test. A survival plot for graft loss stratified for tertiles of skin-AF was constructed from an unadjusted Cox-regression model. Cox regression analysis was used to construct a model for the prediction of graft loss. First, all baseline variables depicted in tables 1 and 2 were entered in univariable Cox regression analyses. All continuous variables showed a linear trend in the estimated hazard ratios, and were thus introduced in the Cox-regression analyses as continuous variables. Subsequently, variables that at least showed a trend (P=0.10) were entered in a multivariable Cox-regression analysis. Variables that did not retain significance were removed from the model, which resulted in the first multivariable model. Next, we (re)introduced known risk-factors for graft loss to validate the multivariable model. This resulted in the final multivariable model which was tested for interaction terms. Diabetes and HbA1c were separately tested for potential interaction. Furthermore, in sub-analyses we investigated the association of skin-AF with all-cause mortality and death-censored graft loss as separate end-points. Log-Minus-log survival curves and time-dependent covariates were used to evaluate adherence of the Cox proportional hazard assumptions. No violations of the proportional hazard assumption were identified. ROC curves were plotted for skin-AF, urinary protein excretion, patient age, hsCRP, and creatinine clearance. For the ROC analyses censoring was ignored by using a fixed follow-up time of 4.4 years for which complete follow-up data was available, as has been described by Mandel et al.[18]

RESULTS

A total of 302 outpatients (age 50 ± 12 years, 45% females, creatinine clearance 63 ± 23 ml/min) participated at a median time of 6.1[2.6-12.1] years after transplantation. Baseline characteristics are summarized in tables 1 and 2 stratified for tertiles of skin-AF. Fifty-one patients (17%) were identified as having diabetes mellitus and 215 patients (71%) as having hypertension. Skin-AF of the leg was slightly higher than skin-AF of the arm (2.9±1.0 vs. 2.6±0.7 a.u., P<0.001). Average skin-AF was 2.7±0.8 a.u. Trend analysis showed that skin-AF was positively associated with patient age, female sex, donor age, dialysis duration, hypertension, smoking, CVD history, systolic blood pressure, glucose concentration, HbA1c, hsCRP, statin use, and inversely with creatinine clearance, and hypercholesterolemia (tables 1 and 2).

Follow-up was performed for a median [interquartile range] time of 5.2 [4.6-5.4] years, during which 53 patients reached the endpoint of graft loss (19 graft failures, 34

Table 1. Recipient characteristics

Characteristics	Tertiles of skin-AF			*P*-value
	1.2-2.3 a.u. n=100	2.3-2.9 a.u. n=101	2.9-5.2 a.u. n=101	
Patient demographics				
Age patient (years)	45.0 ± 11.3	49.3 ± 11.9	57.4 ± 10.4	< 0.001
Sex patient (male)	67 (67.0)	57 (56.4)	48 (47.5)	0.02
Dialysis duration (months)	20.5 [10.0-37.5]	28.0 [14.0-48.0]	29.0 [12.0-57.0]	0.01
Risk-factors CVD				
Diabetes mellitus (n (%))	13 (13.0)	16 (15.8)	22 (21.8)	0.24
Hypertension (n (%))	57 (57.0)	75 (74.3)	83 (82.2)	< 0.001
Hypercholesterolemia (n (%))	65 (65.0)	49 (48.5)	62 (61.4)	0.05
Smoking (n (%))	13 (13.0)	17 (16.8)	27 (26.7)	0.04
CVD history (n (%))	7 (7.0)	12 (11.9)	20 (19.8)	0.02
Physical examination				
Systolic blood pressure (mmHg)	143.7 ± 18.7	148.9 ± 18.9	162.1 ± 26.7	< 0.001
Diastolic blood pressure (mmHg)	88.1 ± 9.7	88.6 ± 9.2	91.0 ± 11.2	0.11
Body mass index (kg/m^2)	25.1 ± 3.6	26.1 ± 4.6	26.0 ± 4.5	0.15
Laboratory examinations				
Glucose (mmol/l)	4.5 [4.1-4.9]	4.6 [4.2-5.1]	4.7 [4.3-5.3]	0.04
HbA1c (%)	6.1 ± 0.9	6.4 ± 1.1	6.7 ± 1.2	< 0.001
Total cholesterol (mmol/l)	5.6 [4.8-6.1]	5.3 [4.8-5.9]	5.6 [5.0-6.3]	0.14
Triglycerides (mmol/l)	1.6 [1.2-2.4]	1.9 [1.5-2.6]	1.9 [1.4-2.7]	0.21
hsCRP (mg/l)	1.3 [0.6-3.4]	1.5 [0.6-3.3]	3.0 [1.2-7.4]	< 0.001
Creatinine (µmol/l)	133 [118-153]	135 [114-172]	139 [110-175]	0.45
Creatinine clearance (ml/min)	71.5 ± 19.3	64.4 ± 23.6	53.6 ± 21.4	< 0.001
Urinary protein excretion (g/24h)	0.2 [0.0-0.4]	0.2 [0.0-0.4]	0.3 [0.0-0.6]	0.07
Skin-autofluorescence				
Skin-AF arm (a.u.)	2.0 ± 0.3	2.6 ± 0.4	3.1 ± 0.6	< 0.001
Skin-AF leg (a.u.)	1.9 ± 0.5	2.7 ± 0.4	3.9 ± 0.8	< 0.001
Average skin-AF (a.u.)	2.0 ± 0.3	2.6 ± 0.2	3.5 ± 0.5	< 0.001
Drug-use				
RAAS blockade (n (%))	34 (34.0)	39 (38.6)	31 (30.7)	0.49
Beta-blocker (n (%))	62 (62.0)	61 (60.4)	71 (70.3)	0.29
Antidiabetic drugs (n (%))	11 (11.0)	11 (10.9)	17 (16.8)	0.36
Anti-platelet drugs (n (%))	16 (16.0)	22 (21.8)	23 (22.8)	0.44
Statines (n (%))	43 (43.0)	61 (60.4)	53 (52.5)	0.05
Immunosuppressive drug				
Prednisolon day dose (mg)	10.0 [7.5-10.0]	10.0 [7.5-10.0]	10.0 [7.5-10.0]	0.55
Calcineurin inhibitors (n (%))	75 (75.0)	81 (80.2)	80 (79.2)	0.64
Mycophenolate mofetil (n (%))	43 (43.0)	37 (36.6)	41 (40.6)	0.65
Azathioprin (n (%))	36 (36.0)	41 (40.6)	28 (27.7)	0.15

Note. Parametric parameters are expressed as mean ± SD; non-parametric parameters are expressed as median [25%-75% IQR]; ordinal parameters are expressed as n(%). Abbreviations: CVD: cardiovascular disease; hsCRP: high sensitivity C-reactive protein; RAAS: renin angiotensin aldosterone system; Skin-AF: skin-autofluorescence.

deaths). Graft survival stratified for skin-AF tertiles is shown in figure 1. Results of univariable and multivariable Cox-regression analyses are summarized in table 3. Skin-AF significantly predicted graft loss in an univariable Cox regression analysis (HR 2.40 [1.75-3.29], P<0.001). Other factors that univariately predicted graft loss included patient age, smoking, systolic blood pressure, HbA1c, hsCRP, serum creatinine,

Table 2. Transplant characteristics

Transplant characteristics	Tertiles of skin-AF			*P*-value
	1.2-2.3 a.u. n=100	2.3-2.9 a.u. n=101	2.9-5.2 a.u. n=101	
Donor demographics				
Age donor (years)	36.8 ± 15.1	35.1 ± 15.6	40.6 ± 14.9	0.03
Sex donor (male)	59 (59.0)	52 (52.5)	51 (50.5)	0.45
Transplantation type				
Living (n (%))	19 (19.0)	14 (13.9)	14 (13.9)	0.51
Cadaveric (n (%))	79 (79.0)	83 (82.2)	82 (81.2)	0.84
Kidney/pancreas (n (%))	2 (2.0)	2 (2.0)	5 (5.0)	0.36
Kidney/liver (n (%))	0 (0.0)	2 (2.0)	0 (0.0)	0.14
Warm ischemia time (min)	36.5 ± 10.9	40.9 ± 17.6	39.4 ± 13.5	0.09
Cold ischemia time (h)	19.6 ± 10.4	20.5 ± 10.1	21.4 ± 10.2	0.42
HLA-AB mismatch (n (%))	75 (75.0)	66 (65.3)	69 (68.3)	0.31
HLA-DR mismatch (n (%))	41 (41.4)	38 (38.4)	35 (35.0)	0.65
Time since transplantation (year)	6.0 [2.9-12.1]	6.5 [3.2-11.9]	6.0 [2.2-12.0]	0.89
Follow-up time (year)	5.2 [4.8-5.4]	5.2 [4.6-5.4]	5.2 [4.2-5.4]	0.15
Acute rejection (n (%))	48 (48.0)	49 (49.5)	36 (35.6)	0.10

Note. Parametric parameters are expressed as mean ± SD; non-parametric parameters are expressed as median [25%-75% IQR]; ordinal parameters are expressed as n(%). Abbreviations: skin-AF: skin-autofluorescence.

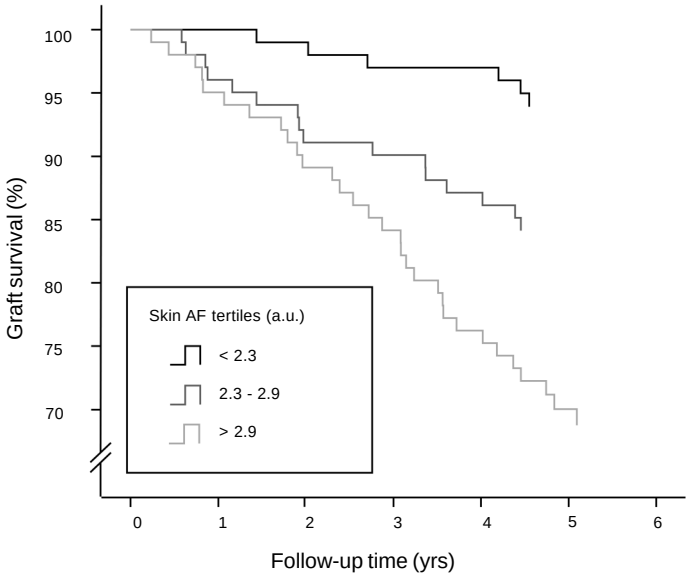

Figure 1. Graft survival stratified for skin-AF tertile
Shown are unadjusted Cox-survival curves stratified on tertiles of skin-AF

Table 3. Results of univariable and multivariable Cox regression analysis

Characteristics	Univariable		Multivariable	
	HR [95% C.I.]	*P*-value	HR [95% C.I.]	*P*-value
Patient demographics				
Age patient (year)	1.04 [1.01-1.06]	0.003	1.03 [1.00-1.06]	0.04
Sex pat (male)	0.98 [0.57-1.69]	0.94		
Dialysis durations (months)	1.03 [0.46-2.27]	0.95		
Donor demographics				
Age donor (year)	1.02 [0.99-1.04]	0.07		
Sex donor (male)	1.31 [0.75-2.26]	0.34		
Risk factors CVD				
Diabetes mellitus (yes)	1.31 [0.67-2.54]	0.43		
Hypertension (yes)	1.85 [0.93-3.67]	0.08		
Hypercholesterolemia (yes)	1.03 [0.46-2.27]	0.95		
Smoking (yes)	2.45 [1.39-4.33]	0.002		
CVD history (yes)	1.03 [0.46-2.27]	0.95		
Physical examination				
Systolic blood pressure (mmHg)	1.02 [1.01-1.03]	0.003		
Diastolic bood pressure (mmHg)	1.03 [0.99-1.04]	0.33		
Body mass index (kg/m^2)	1.02 [0.95-1.08]	0.64		
Laboratory values				
Glucose (mmol/l)	1.00 [0.79-1.27]	0.99		
HbA1c (%)	1.30 [1.05-1.59]	0.01		
Total cholesterol (mmol/l)	0.93 [0.70-1.23]	0.61		
Triglycerides (mmol/l)	1.08 [0.91-1.29]	0.38		
hsCRP (mg/l)	1.03 [1.02-1.04]	< 0.001	1.02 [1.00-1.03]	0.03
Ceatinine (µmol/l)	1.01 [1.01-1.01]	< 0.001		
Creatinine clearance (ml/min)	0.97 [0.96-0.98]	< 0.001	0.99 [0.97-1.0]	0.05
Protein excretion (g/24h)	1.54 [1.33-1.78]	< 0.001	1.57 [1.34-1.83]	< 0.001
Skin-AF (a.u.)	2.40 [1.75-3.29]	< 0.001	1.83 [1.22-2.75]	0.003
Transplant characteristics				
Transplantation type				
Living (yes)	1.14 [0.55-2.33]	0.73		
Cadaveric (yes)	1.02 [0.51-2.03]	0.96		
Kidney/pancreas (yes)	0.59 [0.08-4.27]	0.60		
Kidney/liver (yes)	n/a	n/a		
Warm ischemia time (min)	1.00 [0.98-1.02]	0.95		
Cold ischemia time (h)	0.99 [0.97-1.02]	0.65		
HLA-AB mismatch (yes)	1.01 [0.56-1.82]	0.96		
HLA-DR mismatch (yes)	1.28 [0.74-2.20]	0.38	2.02 [1.14-3.61]	0.02
Time since transplantation (year)	0.98 [0.94-1.03]	0.39		
Acute rejection (yes)	1.24 [0.72-2.12]	0.44		
Drug-use				
RAAS blockade (yes)	0.67 [0.36-1.24]	0.20		
Beta-blocker (yes)	0.82 [0.48-1.43]	0.49		
Antidiabetic drugs (yes)	1.19 [0.56-2.52]	0.66		
Anti-platelet drugs (yes)	1.02 [0.52-1.97]	0.97		
Statines (yes)	1.04 [0.61-1.78]	0.90		
Immunosuppressive drug				
Prednisolon day dose (mg)	1.18 [0.93-1.50]	0.18		
Calcineurin inhibitors (yes)	0.93 [0.49-1.77]	0.82		
Mycophenolate mofetil (yes)	0.89 [0.51-1.55]	0.69		
Azathioprin (yes)	0.81 [0.45-1.45]	0.47		

Note. Table 3 presents the results of univariable and multivariable Cox regression analysis. Hazard ratios are provided with 95% confidence intervals. Abbreviations: CVD: cardiovascular disease; hsCRP: high sensitivity C-reactive protein; RAAS: renin angiotensin aldosterone system; Skin-AF: skin-autofluorescence.

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creatinine clearance, and urinary protein excretion. Furthermore, a trend (*P*=0.10) for an association with graft loss existed for donor age, and hypertension. Variables with at least a trend (*P*=0.10) for an association with graft loss were entered into a multivariable Cox regression analysis. Variables that did not retain significance were subsequently removed from the model, which resulted in a multivariable model for prediction of graft loss consisting of skin-AF (HR 2.34 [1.70-3.24], *P*<0.001), protein excretion (HR 1.51 [1.31-1.75], *P*<0.001), and hsCRP (HR 1.02 [1.01-1.04], *P*=0.003). To further validate this model we (re)introduced known predictors of graft loss. No significant independent contribution was found for patient sex, use of calcineurin inhibitors, diabetes mellitus, HbA1c, glucose concentration, acute rejection, donor age, hypertension, hypercholesterolemia, BMI, and ischemia times. We did, however, find additional contributions of patient age, creatinine clearance, and HLA-DR mismatching to our model, which resulted in a final model consisting of skin-AF (HR 1.83 [1.22-2.75], *P*=0.003), patient age (HR 1.03 [1.00-1.06], *P*=0.04), hsCRP (HR 1.02 [1.00-1.03], *P*=0.03), creatinine clearance (HR 0.99 [0.97-1.00], *P*=0.05), urinary protein excretion (HR 1.57 [1.34-1.83], *P*<0.001), and HLA-DR mismatching (HR 2.02 [1.14-3.61], *P*=0.02). No significant interaction of skin-AF with other predicters of graft loss, including patient age, creatinine clearance, proteinuria, diabetes mellitus, HbA1c, and hsCRP was identified. Finally, sub-analysis revealed that skin-AF was significantly associated with both all-cause mortality (HR 2.50 [1.72-3.64], *P*<0.001), and death-censored graft loss (HR 2.42 [1.43-4.09], *P*=0.001).

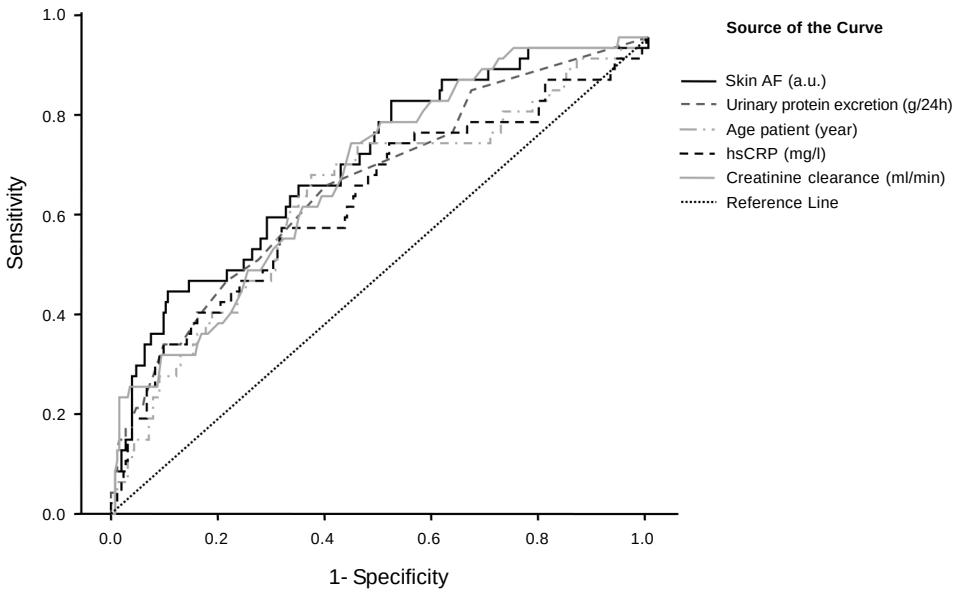

Figure 2. ROC curves for graft loss

Shown are the ROC curves of skin-AF, urinary protein excretion, age patient, hsCRP, and creatinine clearance.

Receiver-Operator-Characteristic (ROC) curves for graft loss are shown in figure 2. The area under the ROC curve of skin-AF (0.73 [0.65-0.81]) was similar to the one for urinary protein excretion (0.69 [0.61-0.78]), patient age (0.66 [0.57-0.75]), hsCRP (0.66 [0.56-0.75]), and creatinine clearance (0.71 [0.63-0.79]). All areas under the ROC curve were significantly different from 0.5.

Discussion

In the present study we showed for the first time that skin-AF, a validated marker for the accumulation of AGEs, is a strong predictor of graft loss in renal transplant recipients. The association of skin-AF with graft loss was independent from other identified risk factors, including patient age, hsCRP, creatinine clearance, protein excretion, and HLA-DR mismatching. The prognostic value of skin-AF for graft loss was comparable to the prognostic value of the other significant predictors of graft loss as was concluded from the area under the curves (AUC) found by the ROC curve analyses.

So far, no prospective study existed which investigated the association of AGEs with graft failure or mortality in kidney transplant recipients. However, some data exist of studies that investigated associations of oxidative stress and AGEs in kidney transplantation. Raj et al[19] investigated levels of circulating AGEs and markers of oxidative stress in patients that had developed chronic renal transplant dysfunction. Patients with biopsy-proven chronic renal transplant dysfunction had higher levels of AGEs and markers of oxidative stress when compared with transplant recipients with normal renal function and patients with chronic renal failure of their native kidneys. Recently, data from our own center showed that inhibition of AGE formation is renoprotective in a Fischer 344 to Lewis (F-L) allograft rat model of experimental chronic renal transplant dysfunction.[20]

Several studies did investigate the association of AGEs with outcome in ESRD. Overall, the findings of these studies have been inconsistent.[21-23] Wagner et al.[21] and Roberts et al.[22] reported that high levels of AGEs are a risk factor for mortality, whereas Schwedler et al.[23] reported a potential protective effect of serum AGEs for mortality. In a non-diabetic population high serum AGE levels were found to be a risk factor for mortality in women but not in men.[24] In a type 2 diabetic population serum AGEs were not found to be a risk factor for cardiovascular mortality.[25] In all of these studies, however, serum levels of AGEs were measured, which are more prone to short-term variations than tissue AGE-accumulation. Our group previously reported that AGE-accumulation measured as skin-AF was associated with mortality in dialysis patients and diabetic patients independent from known risk factors.[11,26] In the present study we confirmed this association in kidney transplant recipients.

Although our data limits us in being conclusive about causality, we can speculate about possible underlying pathways that may explain the prognostic value of AGEs found in the present study. In kidney transplantation oxidative stress may be a source for AGE-accumulation.[27] Oxidative stress itself may be a consequence of ischemia-reperfusion injury, chronic rejection and immunosuppressive therapy.[28-30] Oxidative

stress damages DNA, proteins and lipids via chemical reactions of oxygen and nitrogen radicals. It has been hypothesized that protein damage resulting from oxidative stress such as advanced glycation could be the main contributor for pathological changes in ESRD.[9] Certain damaged proteins may be recognised by pro-inflammatory receptors as it is the case for AGEs and their receptor (RAGE).[31] Under healthy conditions AGEs are cleared efficiently by the kidney without causing severe damage, but under uremic conditions AGEs may accumulate significantly potentially leading to enhanced receptor binding and prolonged pro-inflammatory signaling.[32] The AGE-RAGE interaction stimulates second messenger pathways, among which the renin-angiotensin pathway, the Rac-Cdc42 pathway, the Jac-Stat pathway, and the production of reactive oxygen species via the NADPH oxidase pathway.[7] Besides activation of these pathways the AGE-RAGE interaction also up-regulates nuclear factor-κB (NF-κB) which subsequently up-regulates the production of inflammatory mediators such as TNF and VCAM-1, and also RAGE itself.[33,34] The up-regulation of RAGE and the production of reactive oxygen species may finally lead to a vicious cycle and an amplified inflammatory response. In addition to the activation of receptor-mediated pathways, AGEs can also directly affect endogenous targets. AGEs can covalently bind other AGEs and form cross-links between matrix proteins such as collagen. Extensive cross-linking may then lead to e.g. myocardial stiffening and cardiac mortality.[35]

Although skin-AF has been validated to represent accumulation of AGEs, it has to be taken into account that the fluorescence wavelength used to measure AGEs is not specific. Besides AGEs other substances such as lipofuscin and ceroid exist in the human organism which can be detected using the same excitation and emission wavelengths.[36] However, precursors for the formation of these so-called age pigments and AGEs both result from oxidative stress,[36] which suggests that skin-AF measures the accumulation of oxidative-stress-derived metabolites in general rather than AGEs in particular. Skin-AF might also represent susceptibility for chronic diseases in general rather than a specific susceptibility to renal or cardiovascular disease. Finally, the skin-AF reader has to date only been validated in non-Caucasians, limiting the implications of our results to Caucasian patients.

We were also limited by the number of cardiovascular deaths and graft failures in our study as these were too low to investigate a specific association of skin-AF with cardiovascular mortality or graft failure due to chronic transplant dysfunction. Using cardiovascular mortality instead of all-cause mortality and graft failure due to chronic transplant dysfunction instead of all-cause graft failure as end points would have been more supportive to the general theory of AGE pathology. However, in renal transplant patients most deaths are due to cardio-vascular events, and most graft failures due to chronic transplant dysfunction.[37] Thus, our finding of an association with mortality and graft failure is strongly supportive of a role of AGEs.

The predictive power of skin-AF is not stronger than that of proteinuria or creatinine clearance. However, the practical benefit of skin-AF is that it is a predictor independent of age, proteinuria, hsCRP, and creatinine clearance. Thus, it independently adds to the prognostication of individual patients. Another practical benefit is its simplicity. While proteinuria and creatinine clearance require 24h-collection of urine and

laboratory assessments, and hsCRP requires blood sampling and laboratory assessment, skin-AF can be measured directly at the outpatient clinic within a few minutes, without any inconvenience to the patient.

In conclusion, our data show for the first time that high skin-AF values are strongly and independently associated with the development of graft loss in kidney transplant recipients. Although we should keep in mind that skin-AF is no direct measurement of AGE-accumulation, we do feel that our results are in line with results of other studies and they support the general concept that oxidative stress and AGE-accumulation are patho-physiologically involved the development of graft loss in renal transplant recipient. Skin-AF might be a useful method to estimate the risk for graft loss after kidney transplantation. Further research is needed to investigate, whether AGE lowering therapies could be beneficial for renal transplant recipients.

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Summary

PART I

Advanced glycation end-products (AGEs) are molecules formed during a non-enzymatic reaction between proteins and sugar residues, called the Maillard reaction. AGEs accumulate in the human body with age, and accumulation is accelerated in the presence of diabetes mellitus. In patients with diabetes, AGE-accumulation is associated with the development of cardiac dysfunction. Enhanced AGE-accumulation is not restricted to patients with diabetes, but can also occur in renal failure, enhanced states of oxidative stress, and by an increased intake of AGEs. Several lines of evidence suggest that AGEs may be related to the development and progression of cardiac dysfunction in non-diabetic patients as well.

Chapter 1 described the possible pathophysiological and clinical implications of advanced glycation end-products (AGEs) in heart failure. Basic AGE physiology, and the pathophysiological role that AGEs may play in the development and progression of heart failure were discussed. Next, human and animal studies of the role of AGEs in heart failure were reviewed. Finally, the possible clinical implications of AGE intervention in heart failure were discussed. We concluded that AGEs seem to be a novel and interesting new target in the treatment of chronic heart failure.

AGEs are known to increase in renal failure. Therefore, particularly dialysis patients are subjected to an increased risk for deleterious effects of AGEs. One important effect of AGEs is the modification of collagen by increased cross-link formation leading to tissue stiffening. In the heart this is thought to be related to the development of diastolic dysfunction. Interestingly, diastolic dysfunction is frequently found in dialysis patients. The study described in **chapter 2** aimed to assess whether AGE-accumulation in dialysis patients was related to the presence of diastolic dysfunction. Diastolic function was assessed in 43 dialysis patients using tissue velocity imaging (TVI) on echocardiography. It was compared with tissue AGE-accumulation measured using a validated skin-autofluorescence (skin-AF) reader, and the plasma AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), and pentosidine. Although plasma AGEs were not significantly associated with diastolic function, we showed that skin-AF was strongly and independently associated with diastolic function. We concluded that these results support the concept that tissue AGEs explain part of the increased prevalence of diastolic dysfunction in dialysis patients, but this concept needs to be further established in interventional studies using AGE lowering therapies.

One of the AGE interventions of interest is the use of angiotensin II type 1 receptor antagonists (ARBs). ARBs have shown to be effective in lowering in vitro and in vivo AGE formation. In **chapter 3** it was investigated whether the ARB eprosartan could improve AGE-accumulation and diastolic function in patients with hypertension and diastolic dysfunction. Data were presented from 97 patients who were randomly assigned to 6 months open-label treatment with either eprosartan and other anti-hypertensives or other anti-hypertensives alone. Diastolic function was blindly assessed using echocardiography. Tissue AGE-accumulation was measured using the skin-autofluorescence (skin-AF) reader. Plasma CML and CEL were measured by LC-MS/MS. Plasma pentosidine was measured by HPLC. Results showed that eprosartan use

for 6 months did not result in significant changes in skin-AF and levels of CML, CEL, and pentosidine when compared with control treatment. Additionally, no effects of eprosartan were found on diastolic function. We did show that higher baseline skin-AF was significantly correlated with deteriorating diastolic function. Circulating AGEs showed no strong correlation with changes in diastolic function. We concluded that, irrespective of what antihypertensive drugs were used, patients with lower skin-AF at baseline, seem to benefit more from blood pressure reduction with respect to diastolic function when compared with those with higher skin-AF levels.

The concept that AGEs may be involved in the development of heart failure is supported by studies showing prospective value of AGEs in heart failure. In **chapter 4** the clinical and prospective value of the circulating AGEs CML and CEL were determined in a cohort of 102 chronic heart failure patients. It was shown that CML levels were significantly associated with NYHA functional class and NT-proBNP levels. Moreover, survival analysis for the combined end-point of death, heart transplantation, ischemic cardiovascular event, and hospitalization for heart failure revealed that CML levels predicted outcome, even after adjustment for age, gender, aetiology of CHF, identified risk modifiers, and several known predictors of outcome in chronic heart failure. However, the predictive value of CML subsided after correction for renal function. CEL levels were not significantly associated with the severity and prognosis of heart failure. We concluded that plasma AGEs, in particular CML levels, were related to the severity and prognosis of CHF. Our finding that the relation between CML and prognosis subsided after correction for renal function may suggest that AGE-accumulation in renal failure explains part of the prognostic value of renal function in chronic heart failure.

PART 2

In part 1 of this thesis the possible role of AGEs in the development and progression of heart failure was studied. Part 2 focuses on the putative role of AGEs in the development and progression of chronic renal transplant dysfunction. Chronic renal transplant dysfunction is one of the leading causes of graft failure in kidney transplantation. A complex interplay of both alloantigen-related and alloantigen-unrelated risk factors is believed to underlie its development. In **chapter 5** it is proposed that advanced glycation end-products (AGEs) are involved in the development of chronic renal transplant dysfunction. AGE formation is associated with different alloantigen-unrelated risk factors for chronic renal transplant dysfunction, such as recipient age, diabetes, proteinuria, hypertension, and dyslipidemia. In vitro studies have shown that AGEs induce the expression of various mediators associated with chronic renal transplant dysfunction. Furthermore, AGE-induced renal damage has been found in multiple experimental studies. This renal damage shows similarity to the damage found in chronic renal transplant dysfunction. A summary of all studies available examining the influence of kidney transplantation on AGE-accumulation in blood and tissue showed that although AGE levels were lower in transplant recipients compared with dialysis patients,

levels remained well above normal control levels. We concluded that several lines of evidence support a role of AGEs in the development of chronic renal transplant dysfunction.

AGEs accumulate during renal failure and dialysis. Kidney transplantation is thought to reverse this accumulation by restoring renal function. Results from previous studies showed that this reversal was only partially present. In **chapter 6**, we aimed to confirm these studies in locally obtained data. Using the AGE-reader 285 transplant recipients, 32 dialysis patients, and 231 normal control subjects were evaluated for AGE levels. Results showed that skin-AF levels were significantly increased in dialysis patients compared with normal control subjects, but were lower in transplant recipients when compared with dialysis patients. However, skin-AF levels in transplant recipients were still higher than control patients. These results, like that of others, suggest that kidney transplantation not fully corrects increased AGE levels found in dialysis patients and other factors like e.g. oxidative stress may also play a role in explaining increased AGE levels in kidney transplant recipients.

The aim of the study described in **chapter 7** was to investigate which factors are associated with tissue AGE-accumulation in renal transplant recipients. AGE-accumulation was assessed using the AGE-Reader (skin-AF) in 285 consecutive renal transplant recipients. Furthermore, various transplant- and recipient-related factors of interest were collected. Multivariable analysis showed that skin-AF was positively determined by recipient age, systolic blood pressure, smoking, high-sensitivity C reactive protein, duration of pre-transplant dialysis, and negatively by plasma vitamin C levels, creatinine clearance at baseline, and change in creatinine clearance since one year after transplantation. Taken together, these factors explained 41% of the variance of skin-autofluorescence. We concluded that skin-AF is associated with several risk factors for cardiovascular disease and chronic renal transplant dysfunction, a finding which is in line with the hypothesis that AGEs play a role in the pathogenesis of these conditions in renal transplant recipients. Prospective evaluations should further establish whether skin-AF can be used as a marker for an increased risk of graft loss.

In the final chapter of part 2, **chapter 8**, we evaluated the prospective value of skin-AF in 302 renal transplant recipients. The study population was followed for a median of 5.2 [4.6-5.4] years until the first occurrence of graft loss, i.e. graft failure (re-admission to dialysis or re-transplantation) and all-cause mortality. Skin-AF predicted graft loss in the univariable Cox model and in the multivariable model adjusted for other identified risk-factors, including patient age, hsCRP, creatinine clearance, protein excretion, and HLA-DR mismatching. It was concluded that skin-AF independently predicted graft loss in kidney transplant recipients which supports the hypothesis that the accumulation of AGEs is involved in the development and progression of graft loss.

Discussion and future perspectives

This thesis aimed to study the possible role of advanced glycation end-products (AGEs) in heart failure and chronic renal transplant dysfunction.

PART I

Part 1 focused on the possible role of AGEs in heart failure. Chronic heart failure (CHF) is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. CHF is a major and growing public health problem in the United States and in Europe. Approximately 5 million patients in the US and 10 million in Europe have CHF. The number of CHF deaths has increased steadily despite advances in treatment. CHF results in more than 1 million hospitalizations at a cost of more than 15 billion dollars per year in the United States alone. Despite the magnitude of the problem, most of the available treatment modalities in CHF are not primarily aimed at the underlying pathophysiological processes that occur in the heart and blood vessels of patients with CHF. In this respect, accumulation of advanced glycation end-product (AGEs) might prove an interesting novel target for the treatment of heart failure.

From the pathophysiological aspects of AGEs one would expect that the link between AGEs and heart failure can in part be attributed to the development of diastolic dysfunction. One of the first questions that arose was whether we could establish a relation between AGE-accumulation and diastolic dysfunction. This questions has been extensively investigated in animals,[1-5] however only a limited number of human studies have looked at this issue.[6-8] The study described in chapter 2 aimed to analyse whether diastolic function correlated to AGE measurements.

We chose to perform this study in dialysis patients as they are at an increased risk for AGE-accumulation and the prevalence of diastolic dysfunction is high. We found a strong and independent relation between skin-autofluorescence (skin-AF) and diastolic function. This may suggest that the increased accumulation of AGEs in dialysis patients in part explains the increased prevalence of diastolic dysfunction in these patients.

We could not establish a relation between circulating AGEs and diastolic function. This may suggest that plasma AGE levels do not adequately reflect tissue AGE-accumulation. Plasma AGE levels may have been influenced by dialysis modalities, and absorption from food and smoking. This is supported by the finding that plasma AGEs in these patients were strongly associated with dialysis quality, but not related to traditional determinants of AGE formation like age, and HbA1c. However, the study in chapter 2 was rather small, thus we cannot exclude the possibility that a power issue may explain the lack of correlation found between plasma AGEs and diastolic function. Another limitation to the study presented in chapter 2 concerns the skin-AF method to assess tissue AGE-accumulation. Skin-AF measured in our study was evaluated using the AGE-reader. Although the AGE reader was thoroughly validated against skin tissue biopsies, it remains to be investigated whether tissue AGE-accumulation in skin is correlated to AGE-accumulation in the heart. Therefore, it would be worthwhile to pursue further validation of the AGE reader as a measure of tissue AGE-accumulation in the heart in the near future.

More substantial evidence for a role of AGEs in heart failure should come from intervention studies using AGE lowering therapies. One of the AGE interventions of interest, angiotensin II type 1 receptor antagonists (ARBs), were studied in chapter 3. Eprosartan did not lower AGE-accumulation and did not improve diastolic function in this cohort of hypertensive patients with diastolic dysfunction. However, we did show that higher baseline skin-AF was significantly correlated with deteriorating diastolic function. The latter suggests that irrespective of what antihypertensive drugs were used, patients with lower skin-AF at baseline, seem to benefit more from blood pressure reduction with respect to diastolic function when compared with those with higher skin-AF levels.

Prospective cohort evaluations provide more substantial evidence for a role of AGEs in heart failure. In chapter 4 we analysed the prospective value of the AGEs N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL). We showed that CML was associated with the severity of chronic heart failure and higher plasma CML levels were related to an impaired prognosis. The association with prognosis was independent of several other predictive factors, except for renal dysfunction. Renal function is a clinically significant risk factor for mortality in CHF patients and influences AGE levels.[9-11] Therefore, our results may indicate that CML levels explain (part of) the prognostic value of renal function in patients with CHF. However, due to the observational nature of our study, we could not rule out the possibility that CML acts as a marker for impaired renal function, and as such might have predictive value in CHF. It would be of interest to analyse the predictive value of CML in larger heart failure populations. Currently we are already working on analysing circulating AGE levels in the COACH heart failure cohort.[12] In the COACH study 1023 patients were prospectively followed-up for 18 months, which would enable us to further differentiate between the several parameters that influence outcome in heart failure patients, especially regarding the interaction between renal function and AGE-accumulation.

Convincing evidence for a role of AGEs in heart failure comes from two trials with the AGE cross-link breaker alagebrium (ALT-711) in patients with chronic heart failure.[7,8] ALT-711 or 4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride, is the first of a new class of thiazolium derivatives which break established AGE cross-links between proteins. By cleaving AGE cross-links, ALT-711 has the ability to restore compliance of aged and/or diabetic vascular tissue, and myocardium. Clinical experiences with ALT-711 have been favourable.[7,8] Generally, ALT-711 was safe and well tolerated at doses up to 210 mg twice daily. No differences in incidence or type of adverse events were reported so far in patients treated with ALT-711 versus placebo. Despite encouraging results, both trials with ALT-711 in heart failure patients have an open-label design, and thus leave several questions unanswered.

Recently, we have initiated the BENEFICIAL trial, a double-blind, placebo-controlled, randomised trial evaluating the efficacy and safety of Alagebrium (ALT-711) in patients with chronic heart failure. The primary end-point of this study will be aerobic capacity(VO2max) measured at exercise testing. Secondary endpoints include changes in systolic function, diastolic function, advanced glycation end-products (AGE) measurements in blood and on skin, changes in Minnesota Living with Heart Failure (MLHF) score, NYHA heart failure score, patient's and physician's global assessment,

and NT-proBNP. With this study we are hoping to overcome the limitations of the previous trials with ALT-711 in heart failure.

PART 2

Part 2 focused on the role of AGEs in the development of graft loss in kidney transplant recipients. Over the past 30 years, improvements in the prevention and treatment of acute rejection and opportunistic infection have raised the first-year allograft survival to more than 90%. However, long-term allograft survival has not paralleled the improvement of short-term survival. Approximately half of cadaveric renal allografts are lost within 10-12 years after transplantation.

Leading causes of late allograft loss are patient mortality due to cardiovascular disease and development of chronic renal transplant dysfunction.[13] Although generally, the idea is that both components may well be influenced by AGE-accumulation, chapter 5 discusses the possible role of AGEs in the development of chronic renal transplant dysfunction.[14,15] AGEs are found to be elevated in the presence of risk factors involved in the development of chronic renal transplant dysfunction. In vitro data show that AGEs may stimulate various cells to release mediators that contribute to the renal damage found in chronic renal transplant dysfunction. Based on these findings, we proposed a pathophysiological mechanism of AGE-induced renal tissue damage.

In chapter 6 we evaluated the influence of renal transplantation on levels of AGEs measured as skin-AF. We found that although renal transplantation was indeed associated with a decrease in AGE-accumulation, levels remained well above normal control values. These data suggest that the improvement in renal function after transplantation not fully corrects the increased AGE levels found in pre-transplant patients. Another reason for increased AGE levels after transplantation may be *de novo* creation of AGEs. The latter may be a consequence of enhanced oxidative stress, but also other determinants of AGE-accumulation after transplantation, such as found in chapter 7. We do need to emphasize an important limitation of the study presented in chapter 6. Although the difference in age between dialysis patients, transplant recipients and control was limited, no formal matching was performed. Therefore other factors, like the differences between percentages of diabetics may well have influenced our results. Furthermore, no data was obtained on differences in circulating AGE levels. The latter, however, has been done by several others as was discussed in chapter 5.

Chapters 7 and 8 concern data from the chronic transplant dysfunction (CTD) study. Between August 2001 and July 2003, all adult renal transplant outpatients transplanted in our center, who survived the first year beyond transplantation with a functioning allograft, were invited to participate in the CTD-study. In part of these patients we determined skin-AF. From the cross-sectional analysis presented in chapter 7 we learned that skin-AF was associated with several risk factors for cardiovascular disease as well as chronic renal transplant dysfunction. Interestingly we could also establish a relation between (indirect) markers for oxidative stress (vitamin C) and inflammation (hsCRP) with skin-AF. This finding supports the idea that oxidative stress and inflammation may be involved in the acummulation of AGEs after transplantation .

From the prospective analysis presented in chapter 8 we learned that skin-AF was a predictor of graft loss. The association of skin-AF with graft loss was independent from other identified risk factors, including patient age, hsCRP, creatinine clearance, protein excretion, and HLA-DR mismatching. Furthermore, the prognostic value of skin-AF for graft loss was comparable to the prognostic value of the other significant predictors of graft loss.

Although we should keep in mind that skin-AF is no direct measurement of AGE-accumulation, we do feel that our results are in line with results of other studies and they support the general concept that oxidative stress and AGE-accumulation are pathophysiologically involved the development of graft loss in renal transplant recipient. Skin-AF might be a useful method to estimate the risk for graft loss after kidney transplantation. The benefit of skin-AF is that it is a predictor independent of age, proteinuria, hsCRP, and creatinine clearance. Thus, it independently adds to the prognostication of individual patients. Another practical benefit is its simplicity. While proteinuria and creatinine clearance require 24h-collection of urine and laboratory assessments, and hsCRP requires blood sampling and laboratory assessment, skin-AF can be measured directly at the outpatient clinic within a few minutes, without any inconvenience to the patient.

The studies presented in chapters 7 and 8 provide evidence for a role of AGEs in the development of graft loss. However, definite proof would need to come from intervention studies with AGE lowering drugs. Recently, data from an experimental study performed in our own center for the first time examined the effect of AGE intervention in renal transplantation. Waanders et al[16] studied the influence of inhibition of AGE formation using pyridoxamine in a Fisher 344 to Lewis allograft rat model of experimental chronic renal transplant dysfunction. Fisher to Fisher isografts served as controls. Compared to untreated allografts, pyridoxamine significantly decreased proteinuria, serum creatinine, focal glomerulosclerosis, glomerular macrophage influx, interstitial fibrosis, and interstitial macrophage influx. Moreover, pyridoximine significantly ameliorated tubular accumulation of pentosidine, compared to untreated allografts. These data warrant further investigations using AGE-lowering therapies in transplant recipients.

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Nederlandse samenvatting

DEEL I

Advanced glycation end-producten (AGEs) zijn moleculen die worden gevormd tijdens een niet-enzymatische reactie tussen eiwitten en suiker residuen, welke ook wel de Maillard reactie wordt genoemd. AGEs stapelen in het menselijk lichaam gedurende het ouder worden, hetgeen versneld plaatsvindt in patiënten met diabetes mellitus. Uit wetenschappelijk onderzoek komt naar voren dat bij patiënten met diabetes de stapeling van AGEs gerelateerd is aan het ontstaan van functiestoornissen van het hart. De versterkte stapeling van AGEs blijft niet beperkt tot patiënten met diabetes, maar kan ook optreden bij patiënten met nierinsufficiëntie, een verhoogde staat van oxidatieve stress of bij een verhoogde inname van AGEs. Resultaten van diverse studies suggereren dan ook dat AGEs gerelateerd zouden kunnen zijn aan de ontwikkeling en progressie van functiestoornissen van het hart bij niet-diabetische patiënten.

Hoofdstuk 1 beschrijft de mogelijke pathofysiologische en klinische implicaties van AGEs in hartfalen. Allereerst wordt de basale fysiologie van AGE vorming besproken. Vervolgens wordt ingegaan op de pathofysiologische rol die AGEs kunnen spelen bij de ontwikkeling en progressie van hartfalen. Daarbij worden de verschillende humane en experimentele studies besproken die de rol van AGEs bij hartfalen onderzochten. Aansluitend wordt ingegaan op de mogelijke klinische gevolgen van AGE interventie bij hartfalen. Concluderend wordt gesteld dat AGEs een nieuw en interessant aangrijppunt voor behandeling zouden kunnen zijn bij patiënten met hartfalen.

Het is al langer bekend dat AGEs verhoogt aanwezig zijn bij patiënten met nierfalen. Het is dan ook met name de dialyse patiënt, die wordt blootgesteld aan een verhoogd risico voor de schadelijke effecten van AGEs. Een belangrijk effect van AGEs is de verandering van de extra cellulaire matrix door de toegenomen cross-link vorming welke leiden tot verstijving van weefsel. Men denkt dat deze verstijving in het hart gerelateerd is aan de ontwikkeling van de diastolische disfunctie. Interessant daarbij is het gegeven dat nu juist de dialyse patiënten vaak diastolische disfunctie ontwikkelen. De studie beschreven in **hoofdstuk 2** had als doel om vast te stellen of er een verband is tussen de accumulatie van AGEs in dialyse-patiënten en de aanwezigheid van de diastolische disfunctie. Hiertoe werd de diastolische functie beoordeeld in 43 dialyse patiënten door het meten van de diastolische weefsel snelheden (TVI) met echocardiografie. Deze resultaten werden vergeleken met de weefsel accumulatie van AGEs gemeten met behulp van een gevalideerde huid-autofluorescentie (skin-AF) meter, en de plasma AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) en pentosidine. Hoewel de plasma AGEs niet significant geassocieerd waren met de diastolische functie, lieten de resultaten zien dat de skin-AF sterk en onafhankelijk geassocieerd was met de diastolische functie. Concluderend werd gesteld dat deze resultaten de hypothese ondersteunen dat de mate van AGE stapeling in dialyse patiënten een verklaring vormt voor de toegenomen prevalentie van diastolische disfunctie in deze patiënten groep.

Een van de mogelijkheden om de gevolgen van AGEs tegen te gaan zou kunnen liggen in een behandeling met angiotensine II type 1 receptor antagonisten (ARBs). Van ARBs is aangetoond dat ze zowel in vitro en in vivo het ontstaan van AGEs

kunnen remmen. In **hoofdstuk 3** werd nagegaan of de ARB eprosartan AGE-accumulatie zou kunnen verlagen en diastolische functie zou kunnen verbeteren in patiënten met hypertensie en diastolische disfunctie. Hiertoe werden 97 patiënten gerandomiseerd naar 6 maanden open-label behandeling met hetzij eprosartan gecombineerd met andere anti-hypertensiva of andere anti-hypertensiva alleen. De diastolische functie werd geblindeerd beoordeeld door middel van echocardiografie. Verder werd de weefsel accumulatie van AGEs gemeten met behulp van de huid-autofluorescentie (skin-AF) meter en werden ook de plasma AGEs CML, CEL en pentosidine bepaald. De resultaten toonden aan dat gebruik van eprosartan gedurende 6 maanden niet resulteerde in significante veranderingen in skin-AF, CML, CEL en pentosidine in vergelijking met de controle groep. Daarnaast werden geen effecten van eprosartan gevonden op de diastolische functie. Wel lieten we zien dat een hogere uitgangswaarde van skin-AF significant gecorreleerd was met een verslechtering van de diastolische functie. We vonden geen sterke relaties tussen de circulerende AGEs en veranderingen in diastolische functie. Concluderend werd gesteld dat, onafhankelijk van de gebruikte antihypertensiva, patiënten met een lagere skin-AF uitgangswaarde, meer lijken te profiteren van de verlaging van de bloeddruk met betrekking tot de diastolische functie dan diegenen met een hogere skin-AF uitgangswaarde.

Het concept dat AGEs betrokken zouden kunnen zijn bij het ontstaan en de progressie van hartfalen zou worden ondersteund door studies waaruit een voorspellende waarde voor AGEs blijkt in hartfalen patiënten. In hoofdstuk 4 werd de voorspellende waarde van de circulerende AGEs CML en CEL bepaald in een cohort van 102 patiënten met chronisch hartfalen. CML waarden waren significant geassocieerd met NYHA functionele klasse en NT-proBNP waarden. Bovendien, identificeerde survival analyse voor het gecombineerde eindpunt van dood, harttransplantatie, ischemische cardiovasculaire events, en hospitalisatie voor hartfalen, CML als een belangrijke prognostische factor, zelfs na correctie voor leeftijd, geslacht, etiologie van het hartfalen, geïdentificeerde risico factoren, en verscheidene bekende voorspellers van prognose in patiënten met chronisch hartfalen. De voorspellende waarde van CML verdween echter na correctie voor de nierfunctie. CEL levels waren niet significant geassocieerd met de ernst en prognose van het hartfalen. Er werd geconcludeerd dat plasma AGEs, in het bijzonder CML, geassocieerd zijn met de ernst en prognose van hartfalen en dat het feit dat de relatie tussen CML en prognose verdween na correctie voor de nierfunctie zou kunnen betekenen dat AGE-accumulatie in nierfalen een deel van de voorspellende waarde van de nierfunctie bij chronisch hartfalen zou kunnen verklaren. Uit vervolg onderzoek zal moeten blijken of CML niet slechts een onschuldige marker van de nierfunctie is.

Deel 2

In het eerste deel van dit proefschrift werd ingegaan op de mogelijke rol van AGEs bij de ontwikkeling en progressie van hartfalen. Deel 2 richt zich op de mogelijke rol van AGEs bij de ontwikkeling en progressie van chronische niertransplantaat disfunctie. Chronische niertransplantaat disfunctie is een van de belangrijkste oorzaken van het falen van de graft bij niertransplantatie. Een complex samenspel van zowel allogeen-

afhankelijke en allogeen-onafhankelijke risicofactoren zouden ten grondslag liggen aan het ontwikkelen van chronisch transplantaat disfunctie.

In **hoofdstuk 5** werd gehypothetiseerd dat AGEs betrokken zouden kunnen zijn bij het ontstaan van chronische transplantaat disfunctie. De vorming van AGEs is geassocieerd met verschillende allogeen-onafhankelijke risicofactoren voor chronische niertransplantaat disfunctie, zoals leeftijd, diabetes, proteïnurie, hypertensie en hyperlipidemie. Daarnaast hebben in vitro studies aangetoond dat AGEs de expressie van verschillende mediatoren kunnen induceren die ook gerelateerd zijn met chronische niertransplantaat disfunctie. Bovendien, werd in experimentele studies AGE-geïnduceerde nierschade aangetroffen, welke een grote gelijkenis toont met de schade die gevonden wordt bij chronische niertransplantaat disfunctie. Uit een overzicht van alle beschikbare studies die de invloed van niertransplantatie op AGE-accumulatie in bloed en weefsel onderzochten blijkt dat, hoewel de AGE waarden in het algemeen lager waren bij transplantatie patiënten vergeleken met dialyse patiënten, de waarden nog ver boven de normale controles uitkomen. Concluderend werd gesteld dat verschillende bewijzen suggereren dat AGEs mogelijk een rol zouden kunnen spelen bij het ontstaan van chronische niertransplantaat disfunctie.

AGEs stapelen bij nierfalen en dialyse vanwege een gestoorde klaring. Het lijkt dan ook logisch dat niertransplantatie daar mogelijke een positieve invloed op heeft. Uit de resultaten van eerdere studies blijkt dat dit ook daadwerkelijk zo is, maar wel slechts gedeeltelijk. De studie beschreven in **hoofdstuk 6** bevestigt dit resultaat in een Nederlandse patiënten populatie. Met behulp van de AGE-reader werden 285 transplantatie patiënten, 32 dialyse-patiënten en 231 normale controle geëvalueerd. De resultaten toonden aan dat de skin-AF aanzienlijk verhoogd was in dialyse-patiënten in vergelijking met een normale controle groep, maar wel lager waren in transplantatie patiënten in vergelijking met dialyse patiënten. De skin-AF in de transplantatie patiënten was echter nog steeds hoger dan de controle patiënten. Deze resultaten, evenals die van anderen, suggereren dat niertransplantatie de verhoogde AGE levels gevonden in dialyse patiënten niet volledig corrigeert, en dat dus andere factoren zoals bijvoorbeeld oxidatieve stress ook een rol spelen in het verklaren van de verhoogde AGE levels na niertransplantatie.

Het doel van de studie beschreven in **hoofdstuk 7** was te onderzoeken welke factoren geassocieerd zijn met weefsel accumulatie van AGEs in niertransplantatie patiënten. AGE-accumulatie werd beoordeeld aan de hand van de AGE-reader (skin-AF) in 285 niertransplantatie patiënten. Daarnaast werden verschillende transplantatie-en ontvanger-gerelateerde factoren van belang verzameld. Uit multivariabele regressie analyse bleek dat skin-AF positief bepaald werd door de leeftijd, systolische bloeddruk, roken, CRP, de duur van de dialyse voor transplantatie en negatief door de plasma vitamine C levels, de creatinine klaring bij baseline en de verandering in creatinine klaring sinds een jaar na de transplantatie. Samen verklaarden deze factoren 41% van de variantie van skin-AF. Geconcludeerd werd dat skin-AF gerelateerd was met verschillende risicofactoren voor hart-en vaatziekten en chronische niertransplantataat disfunctie, een bevinding die overeenkomt met de hypothese dat AGEs een rol zouden kunnen spelen in de pathogenese van niertransplantaat disfunctie. Prospective evaluaties moeten

verder vaststellen of skin-AF kan worden gebruikt als een marker voor een verhoogd risico van verlies van de graft.

In het laatste hoofdstuk van deel 2, **hoofdstuk 8**, wordt de voorspellende waarde van skin-AF geëvalueerd in 302 niertransplantatie patiënten. De patiënten werden gevolgd voor een periode van 5,2 [4.6-5.4] jaar tot het eerste optreden van graft verlies, gedefinieerd als het falen van de graft (herstart dialyse of re-transplantatie) of dood (all-cause mortality). Uit multivariabele Cox regressie analyse bleek dat skin-AF een significante voorspeller is van verlies van het transplantaat, zelfs na correcties voor andere risico-factoren voor transplantaat verlies met inbegrip van de leeftijd van de ontvangen, hsCRP, de creatinine klaring, proteïnurie en HLA-DR mismatching. Geconcludeerd werd dat skin-AF een onafhankelijke voorspeller is van verlies van het transplantaat in niertransplantatie patiënten hetgeen de hypothese ondersteund dat AGEs betrokken zijn bij het ontstaan en de progressie van chronische niertransplantaat disfunctie.

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Curriculum Vitae

Jasper W.L. Hartog wordt geboren op 22 augustus 1979 te Deventer. Zijn jeugd brengt hij in Deventer door waar hij in 1997 eindexamen Atheneum doet. Als vervolg start hij met de studie Geneeskunde aan de Rijksuniversiteit Groningen. In 2001 participeert hij aan de Junior Scientific Masterclass, alwaar de basis van zijn enthousiasme voor onderzoek wordt gelegd. Bij de afdeling Nefrologie van het Universitair Medisch Centrum Groningen start hij met een onderzoek naar de rol van versuikerde eiwitten (AGEs) bij het ontstaan van transplantaat falen na niertransplantatie. In 2002 rondt hij zijn doctoraal Geneeskunde af met een scriptie hierover. Er volgen 2 jaar co-schappen. Tijdens deze periode blijft hij actief bezig met het onderzoek bij de afdeling Nefrologie, waaruit een aantal publicaties volgt. In 2004 rondt hij met goed gevolg de opleiding tot arts af. Vervolgens werkt hij een jaar als arts-assistent in het Martini Ziekenhuis Groningen bij de afdeling Cardiologie. Daarna vervolgt hij zijn promotie onderzoek bij de afdeling Cardiologie van het Universitair Medisch Centrum Groningen, waar hij onderzoek doet naar de rol van versuikerde eiwitten (AGEs) bij het ontstaan en de progressie van hartfalen. Hiertoe krijgt hij van de Nederlandse Hartstichting in 2006 de Dekker beurs toegekend. Na zijn promotie in december 2008, zal hij in januari 2009 starten met de opleiding Cardiologie, te beginnen met de vooropleiding Interne Geneeskunde in het Martini Ziekenhuis Groningen.

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