Advanced glycation and inflammatory phenomena in renal transplantion

Gross, Sascha

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in

Renal Transplantation

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Stellingen behorende bij het proefschrift

Advanced Glycation and Inflammatory Phenomena in Renal Transplantation

Sascha Gross

1. Measurement of advanced glycation adds to assessment of the risk for mortality in renal transplant recipients (this thesis).
2. Interaction of kidney function with circulating AGEs is an important factor for the risk assessment in renal transplant recipients (this thesis)
3. The AGE-related risk for mortality is largely independent from inflammation (this thesis)
4. The AGE-related risk for mortality is more likely determined by direct interactions of AGEs than by receptor-mediated pathways (this thesis)
5. The serum albumin-dependent risk for graft failure in renal transplant recipients depends on urinary protein excretion but not on inflammation (this thesis)
6. Serum levels of sRAGE may represent RAGE activity as well as an anti-RAGE potential (this thesis)
7. Low levels of CML being a risk factor for mortality and high levels of CML being protective in renal transplant recipients is a paradoxal finding (this thesis)
8. Even if all good quality research resulted in a high impact factor, a high impact factor still would not always represent good quality research.
9. It`s no measure of health to be well adjusted to a profoundly sick society (Krishnamurti)
10. Pride is pleasure arising from a man's thinking too highly of himself. (Spinoza)

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Contents

Chapter 1

Introduction and Aim of the Thesis

Sascha Gross

1.1 Long-term kidney transplant failure and mortality after renal transplantation

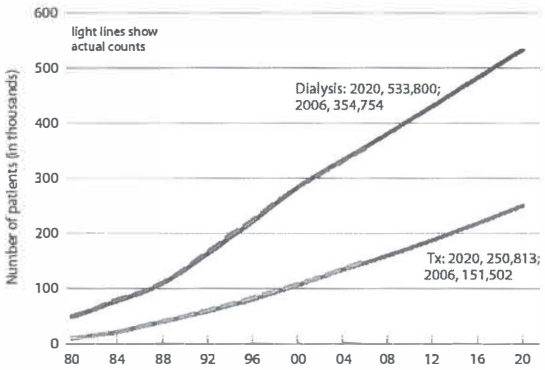

Fig. 1: *Prevalence of dialysis patients and transplant patients in the US. Source: US renal data system annual report 2008.*

The number of end-stage renal disease (ESRD) patients increases all over the world. For example the number of ESRD patients in the US increased dramatically during the 1990ies, from 196,000 in 1991 to 382,000 in 2000(1). The same trends, however lower in total numbers, can be observed in European and other countries(2). Eventually all patients with ESRD will need some sort of replacement therapy, i.e. dialysis or renal transplantation. Projecting the current increase in prevalence and incidence of ESRD showed that the current potential of health care might not be able to meet the future demand of renal replacement therapies in developed countries(1;3). Currently, the demand in developing countries is not met, which results in an additional challenge for facilities in developed countries(2).

Renal transplantation is the preferred replacement therapy, since this treatment has the best outcomes in terms of quality of live and mortality while demanding lower treatment costs(4-6). Although acute rejection after kidney transplantation has largely been eliminated with the application of immunosuppressive drugs, the success of renal trans-plantation is still limited by long-term graft failure and mortality(7). As many as 60% of patients transplanted with a deceased donor kidney develop graft failure within 10 years after transplantation and age-adjusted rates of mortality are approximately four times higher in renal transplant recipients than in the general population(8;9). In renal transplantation the main reason for graft failure is chronic transplant dysfunction (CTD) and the main reason for

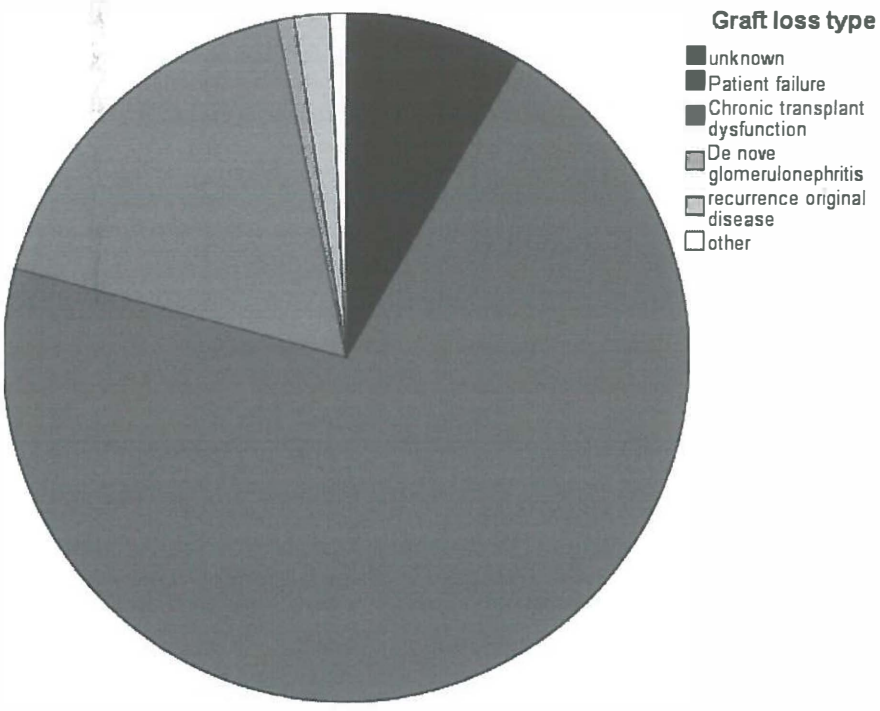

Fig 2: *Causes of graft loss in the Groningen Renal Transplant Database. Chronic Transplant Dysfunction (CTD) accounts for the largest part after patient failure.*

mortality is cardiovascular disease (CVD)(10;11). These trends can also be found from the Groningen Renal Transplant Database, the data of which was subject to this thesis (Fig. 2 and 3).

1.2 Chronic Transplant Dysfunction (CTD)

CTD is a clinical syndrome which consists of many factors. Basically, CTD is defined as progressive renal dysfunction which is independent of acute rejection and specific disease entities, with typical features on biopsy(7). Clinically, CTD is characterized by a gradual decline in renal function which can be measured as increase in serum creatinine in combination with proteinuria and hypertension(12). Histologically CTD is often characterized as chronic allograft nephropathy, which is a descriptive term for a combination of histological changes including interstitial fibrosis, tubular atrophy, atherosclerosis and glomerulosclerosis. Further, the role of inflammation in these lesions is increasingly acknowledged as can be seen from the inclusion of the 'total interstitial inflammation' score into the Banff schema since 2007(13). More recently, also the role of advanced glycation end-products (AGEs) in renal disease became recognized. Interestingly, AGEs have implications in the described lesions and inflammation(14).

1.3 (CVD) Mortality

CVD mortality is the main reason for death after renal transplantation. The main cause for CVD is progression of atherosclerosis. Atherosclerosis is a disease of the large arteries characterized by the accumulation of lipids and fibrous elements. Typically low-density lipoproteins (LDL) are oxidized and damage the vascular wall. Macrophages take up the oxidized LDL and (unable to process it) die and release more oxidized LDL, which attracts more macrophages and so on. As a

consequence the vessel wall becomes hard and narrow. In later stages the lipids accumulate on the vessel wall and a fibrous cap is built over the lipid deposit. Rupture of the fibrous cap ultimately leads to thrombosis and occlusion of the vessel which, in case of coronary arteries, can lead to a heart attack. The role of inflammation in this process is evident. Lately, the role of AGEs has become more and more evident as well(15). AGEs might contribute to the development of atherosclerosis and CVD with involvement of inflammation by receptor-dependent pathways, but AGEs might also contribute without involvement of inflammation(16).

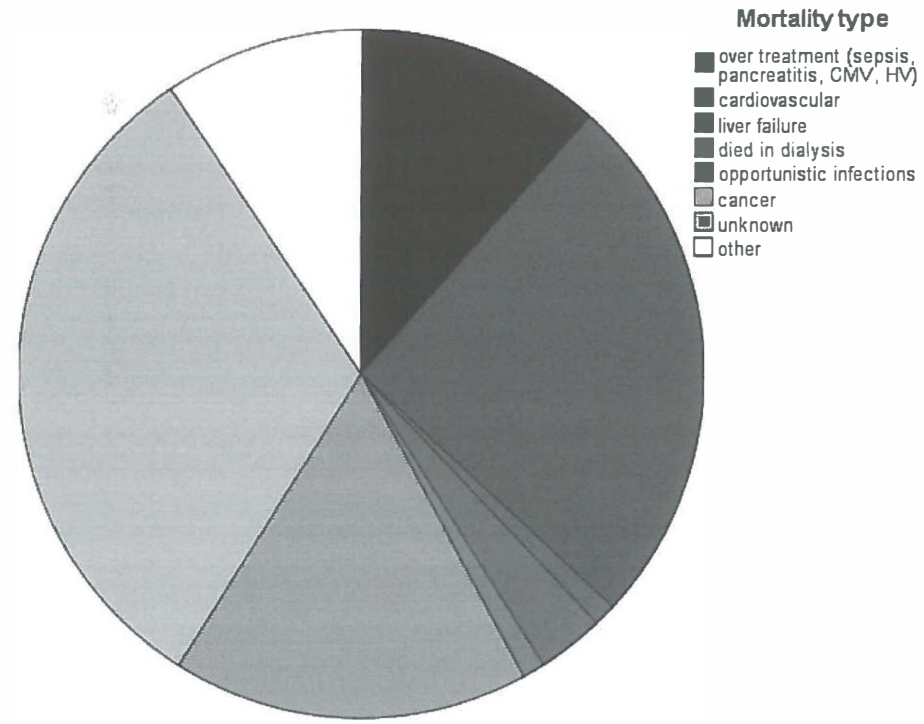

***Fig 3**: Causes of mortality in the Groningen Renal Transplant Database. Cardiovascular mortality accounts for the largest part after unknown causes.*

1.4 AGEs

AGEs are adducts of reducing sugars/ tri-carbonyls and proteins/amino acids(17). They naturally occur at low levels in the human organism, but accumulate in patients suffering from diabetes or uremia(18). Endogenous AGE levels are mainly determined by their intake with food, their endogenous production due to hyperglycemia or oxidative stress and their elimination by the kidney(17;19-21). Accumulation of AGEs has been held responsible for several diseases such as diabetic complications and Alzheimer's Disease(22). More recently evidence has grown for a detrimental role of AGEs in heart and kidney disease(14;23). The pathomechanisms by which AGEs might exert their toxic effect are diverse. In general, they can be divided in receptor-dependent and receptor-independent mechanisms. Receptor-dependent mechanisms include activation of the pro-inflammatory receptor for AGEs (RAGE), which may lead to sustained inflammation and thus vascular complications(21). Receptor-independent mechanisms include cross-linking of LDL-proteins, which results in lower clearance of LDL and thus enhanced risk for atherosclerosis(24). Further, it was suggested that AGEs cross-link extra-cellular matrix proteins of the arterial wall. This may e.g. lead to vascular stiffness and hypertension. As a result vessels are more vulnerable and there is an enhanced risk for atherosclerotic plaques to rupture(22).

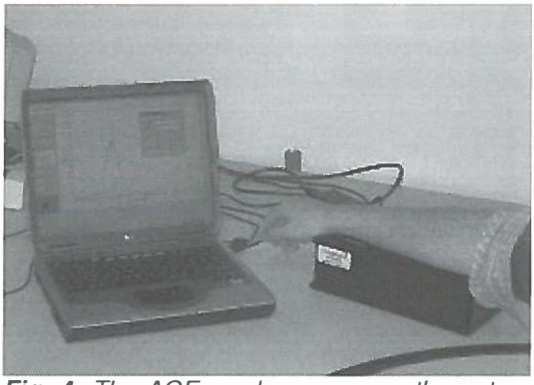

Fig. 4: *The AGE reader measures the auto-fluorescence of skin from the inner side of the forearm.*

AGEs have traditionally been detected using their fluorescence properties in collagen cross-links(25). Collagen is also the major protein occurring in skin. Autofluorescence of the skin has been identified to be associated with endogenous AGE accumulation(26). Skin autofluorescence measurement might therefore be a potent means to measure the risk for heart or kidney failure. Recently a method has been validated to non-invasively measure AGE accumulation by determining skin autofluorescence(27). This method was used in this thesis (chapter 3) to investigate the prospective potential of fluorescent AGEs on graft failure and mortality.

The most abundant and best characterized AGE in humans, Nε-carboxymethyl lysine (CML), however, is not fluorescent(18). AGEs such as CML have widely been measured using antibodies specific for the glycation bond. However, these strategies are limited by several factors: The specificity of the antibody might be difficult to define, endogenous molecules can competitively interact with the AGE-antigen, and proteins that are used to block non-specific interactions may contain AGE-antigens(28). In chapter 4, therefore, CML was measured by liquid chromatography and mass spectrometry from patient serum in order to investigate the prospective potential of CML on graft failure and mortality.

1.5 RAGE

AGEs can bind and activate several cell-surface receptors including the receptor for AGEs (RAGE)(29;30). RAGE elicits a pro-inflammatory response in the endothelium of blood vessels upon activation by AGEs (31). Importantly, this response includes an up-regulation of RAGE itself and may therefore lead to a vicious cycle resulting in chronically sustained inflammation(32). The enhanced inflammation may then further enhance oxidative stress and thus the formation of AGEs(20). The potential pathways of AGE/RAGE pathophysiology in uremia were summarized and an important role was hypothesized for the development of heart failure and chronic kidney transplant dysfunction(14;23).

Besides the full-length receptor also several c-terminal truncated isoforms and one y-terminal truncated isoform were identified (33-36) (Fig. 5). The c-terminal truncated isoforms lack the trans-membrane and signaling domains. As a consequence these isoforms are soluble and occur in several tissues including the blood circulation. These isoforms are commonly referred to as sRAGE and are likely to function as decoy receptors, i.e. they competitively bind RAGE ligands and prevent RAGE-mediated pro-inflammatory signaling. Indeed, in murine models application of sRAGE was found to counteract RAGE-related pathogeneses such as atherosclerosis and decreased wound healing (37;38). In humans also some evidence exists for a potential protective effect of sRAGE: e.g. low serum levels of sRAGE are associated with higher incidence of coronary artery disease in non-diabetic men(39) and high sRAGE levels were associated with extreme longevity(40). In chapter 2 of this thesis sRAGE was measured from patient serum and the potential prediction of serum sRAGE was investigated for graft failure and mortality.

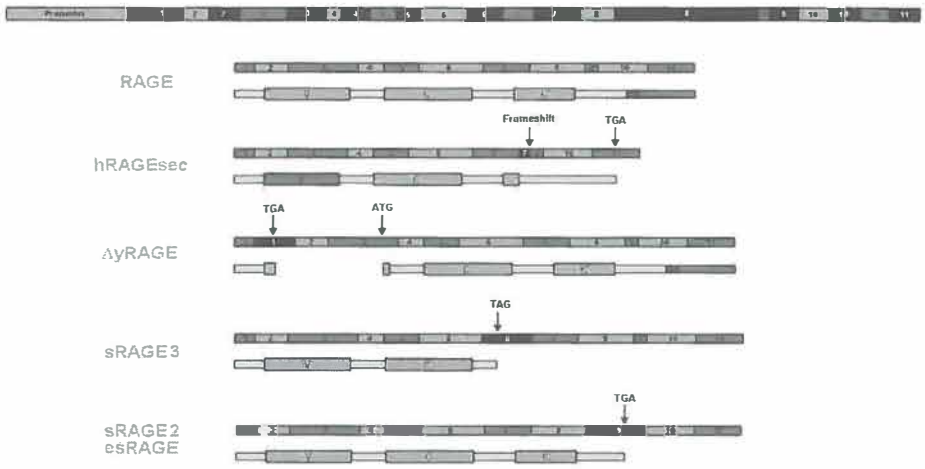

Fig. 5: *RAGE variants. The upper bar resembles the RAGE gene with introns in black and exons in grey. The lower bars resemble the RAGE mRNA and protein domains. Proteins domains: V = variable, C = constant, black = transmembrane, dark grey =cytosolic.*

Besides AGEs other endogenous proteins are able to bind to RAGE. Amongst them are amphoterin (HMGB1), proteins of the S100 family, and Mac-1. HMGB1 is associated with neuronal development(41). Mac-1 is an integrin, which directs inflammatory cells through the vascular wall. S100 proteins are associated with inflammation(42). It has been shown that RAGE is able to prolong the inflammatory response through Mac-1 as well as S100 proteins(43;44). Therefore, it may be possible that RAGE is able to prolong the inflammatory response in renal transplant recipients independent of AGEs. S100B is the most prominent member of the S100 family and has been linked to neuronal damage and inflammation(45). Originally thought to be brain-specific, more recently S100B was also identified in other tissues(46). Moreover, S100B served as a predictive marker for mortality after cardiac surgery and brain trauma(47;48). In chapter 5, therefore, S100B was investigated in order to gain a first insight into a potential role of RAGE-mediated inflammation independent of AGEs.

1.6 Inflammation

Chronic rejection develops in grafts that undergo intermittent or persistent damage from cellular and humoral responses resulting from indirect recognition of alloantigens(12). These responses are typically paired with inflammation and the production of cytokines such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), or transforming growth factor beta (TGF-ß), which have been investigated as inflammatory markers for the prediction of CVD in renal transplant recipients(49). Besides these cytokines the consideration of the acute phase protein c-reactive protein (CRP) as inflammatory marker has shown promising results in the prediction of graft failure and mortality in renal transplant recipients(50). Besides CRP, however, other acute phase markers might be promising as well.

Serum albumin might be one such marker. Besides its function as toxin remover, albumin is also a negative acute phase protein, and low albumin levels may therefore reflect ongoing chronic low-grade inflammation(51;52). Low serum albumin has been shown to be a predictor for both graft failure and mortality in renal transplant recipients(53;54). Mechanisms that have been suggested to underlie this association include chronic low-grade inflammation and proteinuria(53;55). To date it is not clear whether proteinuria or inflammation could be confounders for the development of graft failure or mortality in renal transplant recipients. Inflammation lowers the production rate of albumin and proteinuria enhances the clearance of albumin from the blood. Therefore, both factors could contribute to low albumin levels.

1.7 AGEs RAGE and inflammation

In summary, at least three principles can be hypothesized how AGEs and RAGE could mediate graft failure and mortality: 1) AGEs induce inflammation through RAGE, 2) inflammation is induced by RAGE independent of AGEs, and 3) AGEs exert their effects directly i.e. independent of RAGE. For all three principles potential mechanisms have been identified (24;31;43).

1.8 Aim(s) of the thesis

The general aim of this thesis is to investigate the relation of advanced glycation, inflammation, kidney graft failure and mortality. Thereby, the focus is on the role of AGEs/RAGE as potential enhancers of the inflammatory response. All studies have been performed in kidney transplant recipients, which have decreased kidney function and are therefore more prone to the potentially detrimental effects of AGEs. It was hypothesized that markers related to AGEs, RAGE and inflammation would predict graft failure and mortality in these patients.

In **chapter 2** the role of sRAGE in kidney transplant recipients is investigated. As mentioned before sRAGE might serve as a decoy receptor for RAGE and therefore protect from the detrimental effects of RAGE. In this study it is investigated which factors determine serum sRAGE levels. Also, a negative association of serum sRAGE levels with the risk for mortality or graft failure is hypothesized: an association of sRAGE with outcome could reflect silencing of RAGE and depletion of AGEs.

For **chapter 3** skin auto-fluorescence (skin AF) has been measured in renal transplant recipients. Skin AF has been shown to reflect AGE accumulation in the skin(56). Skin AF could reflect both direct effects of AGEs and RAGE-mediated pathologies. As AGEs have been implicated a role in CTD and CVD we hypothesize that skin AF would predict the development of graft failure and mortality. A significant association would support the idea that long-term AGE exposure has a detrimental role in kidney transplant recipients.

In **chapter 4** Nε-carboxymethyl-lysine (CML) was measured by mass spectrometry from patient serum. In contrast to Chapter 3 serum CML levels reflect a more "acute" impact of serum AGEs rather than a cumulative one. CML is the most prominent AGE and has been investigated as prospective marker for mortality in haemodialysis patients(57). In this chapter it is hypothesized that CML would predict mortality (and graft failure) in renal transplant recipients. Rejecting the null hypothesis would indicate that acute levels of circulating AGEs are risk factors for kidney transplant recipients.

For **chapter 5** S100B has been measured by ELISA. S100B is an endogenous ligand of RAGE and a prominent brain damage marker. S100 proteins have been shown to activate RAGE(43). S100B may therefore reflect RAGE activation without involvement of AGEs. As potential initiators of chronic inflammation S100B may, however, predict mortality and graft failure by activating the RAGE axis. Finding an association of S100B with outcome would support the importance of RAGE-activation after kidney transplantation.

In **chapter 6** serum albumin is investigated. Serum albumin is a negative acute phase protein, and hypoalbuminemia may reflect ongoing chronic low-grade

inflammation(51;52). However, serum albumin does not have a direct relation with AGEs and might reflect non-AGE-related inflammation. Therefore, serum albumin and CRP are in the focus of this study and a prediction of graft failure and mortality by serum albumin levels is hypothesized. Finding an association of serum albumin with graft failure or mortality which depends on CRP would support the importance of chronic low-grade inflammation after renal transplantation.

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Chapter 2

Low levels of sRAGE are associated with increased risk for mortality in renal transplant recipients

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2.1 Abstract

BACKGROUND: Advanced glycation end-products (AGEs) have been implicated in the pathogenesis of atherosclerosis and allograft dysfunction in renal transplant recipients. Infusion of the soluble form of the receptor for advanced glycation end-products (sRAGE) was protective against atherosclerosis and nephropathy in animal models. In this study we investigated determinants of endogenous sRAGE in renal transplant recipients and whether sRAGE was associated with mortality and graft loss.

MATERIALS & METHODS: Patients who were transplanted at our centre and who visited our outpatient clinic were invited to participate in this study between August 2001 and July 2003. Baseline was the date of their first visit, which was at least one year after the transplantation. Follow-up time was the period from baseline until death or admission to dialysis or re-transplantation.

RESULTS: 591 patients participated at a median time of 6 years after transplantation. Independent determinants of sRAGE were mycophenolate mofetil medication ($\beta = -0.21$, $p < 0.001$), creatinine clearance ($\beta = -0.15$, $p < 0.001$), BMI ($\beta = -0.12$, $p = 0.003$) and fasting insulin concentration ($\beta = -0.14$, $p = 0.001$). Low sRAGE levels were associated with a 2-3 times higher risk for mortality especially after correction for creatinine clearance ($p = 0.006$).

CONCLUSION: A lack of sRAGE is a risk factor for mortality in renal transplant recipients. The putatively protective role of sRAGE and its inverse associations with creatinine clearance, mycophenolate mofetil medication, BMI and insulin need further investigation.

2.2 Introduction

Renal transplantation is the preferred treatment for most patients with end stage renal disease, both in terms of quality of life and patient survival(1;2). One-year graft survival has steadily improved from approximately 40% in the seventies, to almost 90% between 1998 and 2001(3). Improvements in long-term graft survival, however, still strongly lag behind, especially if improvements in one year survival are taken into account(4). Main reasons are graft failure due to chronic allograft nephropathy and patient mortality due to accelerated atherosclerosis(5). Age-adjusted rates of mortality are approximately 3-5 times higher in renal transplant recipients than in the general population(6), with cardiovascular disease (CVD) accounting for more than 50% of all deaths(7).

Toxic effects of advanced glycation end-products (AGEs) have been implicated in both accelerated atherosclerosis(8) and chronic allograft nephropathy(9) and enhanced plasma levels of AGEs have been found in patients after kidney transplantation(10). AGEs are supposed to exert their toxic effects in part through cross-linking of proteins and in part through eliciting a pro-inflammatory response upon binding to the receptor for advanced glycation end-products (RAGE)(11). The AGE-RAGE interaction has been hypothesized to activate the redox-sensitive transcription factor NFκB(12), while NFκB itself is known for its role in inflammation and activation of NFκB is implicated in the pathophysiology of several chronic diseases, including atherosclerosis(13) and chronic kidney disease(14). Soluble isoforms of the receptor (sRAGE) have been shown to be able to antagonize the development of diabetic complications, including nephropathy, disturbed wound-healing and accelerated atherosclerosis, in several animal experimental models(15-19), and a specific soluble isoform (endogenous secretory RAGE or esRAGE) has been shown to predict cardiovascular mortality in dialysis

patients(20). High circulating sRAGE concentrations may therefore protect against the toxic effects of AGEs.

The present study aims to investigate cross-sectionally which factors determine circulating sRAGE concentrations in renal transplant recipients, and prospectively whether circulating sRAGE concentrations predict graft loss and mortality in this population.

2.3 Materials & Methods

2.3.1 Study design and patients

The current study was part of a larger prospective study, which was incorporated in the Groningen Renal Transplant Outpatient Program, and details of which have been published previously(21;22). The Institutional Review Board approved the study protocol (METC 01/039), which was in adherence with the Declaration of Helsinki. Between August 2001 and July 2003, all adult allograft recipients who survived the first year after transplantation (1 year post- transplant was considered baseline) with a functioning allograft were eligible to participate at their next visit to the outpatient clinic. A total of 606 out of 847 (72%) eligible renal transplant recipients signed written informed consent. Funding sources had neither a role in the collection and analysis of data, nor in the submission and publication of the manuscript.

2.3.2 Measurements

Blood was drawn after an 8-12h overnight fasting period. Plasma sRAGE levels were determined using the Quantikine® human RAGE ELISA kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). This test employs a mouse monoclonal antibody with an undefined binding epitope on the human RAGE protein (e-mail communication with the R&D systems customer service), i.e. this test only gives information about the quantity of soluble RAGE proteins but not about which isoforms are detected.

The samples had been stored frozen at -80°C in a refrigerator with continuous temperature registration and an automatic temperature alarm. Temperature had never risen to higher than -70°C until assessment. No samples for sRAGE determination were available in 15 cases, leaving 591 subjects eligible for our study. Fasting insulin was determined on an AxSym auto-analyzer (Abbott Diagnostics, Hoofddorp, The Netherlands). Total cholesterol was determined using the CHOD PAP method (MEGA AU 510, Merck Diagnostics, Darmstadt, Germany). Low density lipoprotein was calculated using the Friedewald formula. High density lipoprotein cholesterol was determined using the CHOD PAP method on a Technikon RA-1000 (Bayer Diagnostics B.V., Mijdrecht, The Netherlands). Serum creatinine concentrations were determined using the Jaffé method, serum triglycerides were determined with the GPO-PAP method (both on a MEGA AU 510, Merck Diagnostics, Darmstadt, Germany). HbA_{1c} was determined by HPLC (VARIANT™ HbA_{1c} program with Bio-Rad CARIANT Hb Testing System, Bio-Rad, Hercules, CA, USA).

All patients were instructed to gather a 24h urine collection. Samples of these collections were used to determine protein and creatinine concentrations. Urinary

protein was analyzed using the Biuret reaction (MEGA AU 510,' Merck Diagnostics, Darmstadt, Germany), and urinary creatinine was determined using the Jaffé method on the same equipment as the serum creatinine was determined. Creatinine clearance was calculated from 24h urinary creatinine excretion and serum creatinine concentrations.

The body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (measured to the nearest 0.5 kg and 0.5 cm respectively). Waist circumference was measured twice on bare skin midway between the 10th rib and the iliac crest and the mean of the two measurements was calculated. Blood pressure was measured as the average of three automated (Omron M4; Omron Europe B.V., The Netherlands) measurements with 1 minute intervals after a 6 minutes rest in supine position.

2.3.3 Recipient and transplant characteristics

Relevant donor, recipient and transplant characteristics were 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 donor and recipient age and sex, date of transplantation, delayed graft function (days of oliguria), weight, renal function at baseline, type of acute rejection treatment, and use of medication. Prior history of cardiovascular disease was obtained from a self-report questionnaire which had been sent to participants by mail. Patients were considered to be diabetic if fasting glucose concentration was ≥ 7 mmol/l or if they were using anti-diabetic medication.

Standard immunosuppressive therapy consisted of the following: from 1968 until 1989, prednisolone and azathioprine (100 mg/day; from January 1989 to February 1993, cyclosporine standard formulation (Sandimmune, Novartis; 10 mg/kg; through levels of 175-200 µg/l in first 3 months, 150 µg/l between 3 and 12 months post-transplant, and 100 µg/l thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day). From March 1993 to May 1996, cyclosporine micro-emulsion (Neoral; Novartis Pharma, Arnhem, The Netherlands; 10 mg/kg; trough levels idem) and prednisolone and from May 1996 to date, mycophenolate mofetil (Cellcept; Roche B.V., Woerden, The Netherlands; 2 g/day) was added. Current medication was extracted from the medical record.

2.3.4 Follow-up, mortality and graft loss

All participating patients visited the outpatient clinic at least once a year. Duration of follow-up was calculated as the difference between the baseline visit and the last visit to the outpatient clinic. Follow-up for patients who died with a functioning graft was calculated using the date of the last visit to the outpatient clinic prior to death. Follow-up for patients with graft loss was calculated using the date of the last visit to the outpatient clinic prior to start of dialysis.

2.3.5 Statistical analyses

Analyses were performed with SPSS version 12 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation, whereas non-parametric variables are given as median (interquartile range), and dichotomous variables are given as percentage of the true value. Plasma sRAGE concentration data were categorized into quartiles for univariate and survival analyses. For linear

regression analyses, their distribution was normalized by logarithmic transformation. For all analyses a p-value < 0.05 was considered to indicate significance, except for interaction terms, for which a p-value < 0.10 was considered the threshold. Our analyses can be divided into two parts: First, we performed cross-sectional analyses to investigate determinants of plasma sRAGE levels. Second, we performed prospective analyses to investigate the association of baseline plasma sRAGE with graft loss or mortality.

In the cross-sectional part of our study groups of putative determinants of sRAGE were analysed over quartiles of sRAGE using analysis of variance (ANOVA) for parametric variables, the Jonkheere-Terpstra test for non-parametric variables, and the χ^2-test for dichotomous variables. All variables which showed an association with sRAGE with a p-value < 0.10 were considered for inclusion in further multivariate regression analyses. However, if members of groups were highly correlated to each other ($r \geq 0.50$), only the variables with the strongest relation to sRAGE were included in order to prevent co-linearity and over-adjustment in these analyses. Included variables were analyzed in a backward multivariate linear regression analysis in order to allow for assessment of the effect of adjustments. The effect of adjustment was judged by comparing the (standardized) regression coefficients and p-values of an association before and after adjustment. For this stepwise backward analysis we used $p > 0.05$ as exclusion and $p > 0.01$ as inclusion criterion.

In the prospective part of our analyses we applied Cox-regression models separately for mortality and death-censored graft loss with quartiles of sRAGE as determinants. For both we established 5 survival models: First, including quartiles of sRAGE alone (crude), second, additionally including patient sex, donor sex, patient age, and donor age (Model A), third, additionally including creatinine

clearance (Model B), fourth, additionally including other independent determinants of sRAGE (Model C), and fifth, additionally including other potential determinants relevant for graft loss or mortality, namely diabetes, history of myocardial infarction, serum CRP concentration, and HDL-cholesterol (Model D). Potential existence of a curvilinear relationship between sRAGE and end-points was tested in cox-regression models in which sRAGE and a quadratic term of sRAGE were included as continuous variables.

2.4 Results

A total of 591 outpatients (age 51 ± 12 years, 45% females, creatinine clearance 62 ± 22 ml/min) participated at 6.0 (2.6 – 11.4) years after transplantation in the baseline measurements of our study. Plasma concentrations of sRAGE were 1676 (1234 – 2247) pg/ml. Recipient and transplant characteristics for quartiles of sRAGE are shown in table 1. Concentrations of sRAGE increased significantly with increasing time since transplantation, total cholesterol, LDL-cholesterol, cold ischemia time, serum creatinine, urinary protein excretion, and use of azathioprine. Concentrations of sRAGE decreased significantly with increasing BMI, waist circumference, fasting insulin concentrations, creatinine clearance, daily prednisolone dose, use of mycophenolate mofetil, and CRP concentration.

Table 1: Baseline characteristics over quartiles of sRAGE plasma concentration

	Quartiles of sRAGE				
	I	II	III	IV	*p*-value
n	147	148	148	148	
sRAGE range (pg/ml)	386-1230	1231-1675	1676-2240	2241-6750	
Demographics					
Age recipient (yrs)	51.9 ± 11.8	51.8 ± 11.3	51.1 ± 12.9	51.3 ± 12.4	0.94
Age donor (yrs)	37 ± 16	36 ± 14	37 ± 15	38 ± 16	0.72
Male sex recipient (%)	55.8	60.8	54.7	47.3	0.13
Male sex donor (%)	47.6	59.5	53.7	58.2	0.16
Time since transpl. (yrs)	3.9 (1.6-7.9)	6.1 (2.6-12.5)	6.4 (3.1-12.2)	7.5 (4.2-12.2)	<0.001

	I	II	III	IV	*p*-value
Body composition recipient					
BMI (kg/m²)	27.3 ± 4.2	26.2 ± 4.3	25.6 ± 4.4	25.2 ± 4.0	<0.0001
Waist circumf.(cm)	101 ± 13	98 ± 14	96 ± 14	94 ± 13	<0.001
Smoking recipient (%)					
Current (%)	19.0	20.3	21.6	27.0	0.36
Never (%)	36.7	34.5	34.5	35.1	0.97
Hypertension parameters					
SBP (mmHg)	153.7 ± 23.3	150.8 ± 23.3	152.6 ± 23.2	154.9 ± 24.8	0.91
DBP (mmHg)	90.4 ± 9.1	89.8 ± 9.8	89.9 ± 10.3	89.6 ± 10.5	0.47
Antihypertens. med. (%)	92.5	89.9	82.4	83.1	0.07
No. of anithypertensives	2.07 ± 1.01	1.96 ± 1.16	1.78 ± 1.17	1.80 ± 1.22	0.09
ACEi (%)	32.7	29.1	25.7	23.0	0.27
β-blocker (%)	65.3	63.5	60.8	57.4	0.53
Hyperglycaemia parameters					
Glucose (mmol/l)	4.6 (4.2-5.1)	4.5 (4.0-5.0)	4.5 (4.1-5.0)	4.5 (4.1-5.0)	0.60
Insulin (µU/ml)	13.0 (9.5-18.9)	11.3 (7.8-15.7)	10.5 (8.0-14.5)	10.3 (7.2-14.1)	<0.001
Diabetes Mellitus (%)	23.1	16.9	14.2	17.6	0.24
HbA_{1c} (%)	6.7 ± 1.1	6.5 ± 1.1	6.4 ± 1.0	6.5 ± 1.1	0.09
Use of anti-diabetic (%)	16.3	12.8	9.5	14.9	0.34
History of CVD					
Prior MI (%)	7.6	9.5	8.1	7.5	0.92
Use of antiplatelet drugs (%)	21.8	21.6	17.6	17.6	0.66
Lipid parameters					
Total chol. (mmol/l)	5.4 (4.8-6.1)	5.6 (4.8-6.1)	5.6 (5.0-6.2)	5.8 (5.2-6.6)	0.001
HDL chol.(mmol/l)	1.0 (0.9-1.2)	1.0 (0.8-1.3)	1.1 (0.9-1.3)	1.1 (0.9-1.4)	0.18
LDL cholesterol (mmol/l)	3.5 (2.8-4.0)	3.5 (2.9-4.0)	3.5 (3.0-4.2)	3.6 (3.0-4.3)	0.03
Triglycerides (mmol/l)	1.9 (1.3-2.6)	1.9 (1.4-2.6)	2.0 (1.4-2.7)	1.9 (1.6-2.7)	0.14
Use of statins (%)	49.0	51.4	45.9	52.0	0.72
History of kidney failure					
Previous transplant. (%)	6.8	8.8	10.1	14.9	0.13
Prior dialysis (months)	33 (17-51)	28 (13-47)	26 (11-47)	25 (12-56)	0.24
Allograft viability					
No dead donor (%)	19.0	11.5	12.8	10.1	0.12
Warm ischemia (min)	35 (30-43)	36 (30-45)	35 (30-45)	36 (30-46)	0.21
Cold ischemia (h)	20 (13-24)	23 (16-28)	20 (14-27)	24 (18-28)	0.004

	I	II	III	IV	*p*-value
Allograft function					
Serum creat.(µmol/l)	130 (109-150)	132 (110-165)	132 (111-159)	146 (120-187)	0.001
Creat. clear. (ml/min)	66 ± 21	64 ± 22	62 ± 21	55 ± 23	<0.001
Urinary prot. excr. (g/24h)	0.2 (0.0-0.4)	0.2 (0.0-0.5)	0.2 (0.0-0.5)	0.3 (0.1-0.7)	0.02
Acute rejection treatment					
Corticosteroids (%)	35.6	43.9	38.2	41.2	0.50
Antilymphoc. AB's (%)	18.5	15.5	12.2	10.8	0.23
HLA mismatches					
HLA-AB mismatch (%)	70.7	70.9	74.3	74.3	0.49
HLA-DR mismatch (%)	40.3	40.0	36.8	30.3	0.35
Immunosuppression therapy					
Prednisolone dose (mg/d)	10 (10-10)	10 (7.5 -10)	10 (7.5-10)	10 (7.5-10)	0.007
Ciclosporine (%)	69.4	60.8	64.9	62.2	0.43
Tacrolimus (%)	17.0	14.2	14.9	10.1	0.39
Mycophen. mofetil (%)	51.7	46.6	39.2	25.0	<0.001
Azathioprine (%)	25.2	32.4	31.8	41.9	0.02
Inflammation					
CRP (mg/l)	2.6 (1.1-5.7)	1.9 (0.9-4.2)	1.7 (0.6-4.7)	2.1 (1.0-5.0)	0.05

The results of the stepwise backward multivariate linear regression analyses of independent factors associated with sRAGE are shown in table 2. Of the potential determinants, BMI and waist circumference were strongly correlated ($r = 0.81$, $p < 0.001$), with BMI having a stronger relation with sRAGE than waist circumference. Total cholesterol was strongly correlated with LDL-cholesterol ($r = 0.84$, $p < 0.001$), creatinine clearance with serum creatinine ($r = -0.61$, $p < 0.001$), and use of mycophenolate mofetil with use of azathioprine ($r = -0.58$, $p < 0.001$), with the respective first parameters in these three correlations having the strongest relations with sRAGE. BMI, fasting insulin concentration, creatinine clearance, and use of mycophenolate mofetil remained in the final backward multiple regression analysis, and were therefore considered independent determinants of sRAGE. The association of sRAGE with time since transplantation disappeared from the model because of shared variance with use of mycophenolate mofetil. The same was true

for daily dose of prednisolone and cold ischemia duration. CRP concentration and HbA1c turned out not to be significant in the linear model.

Table 2: Backward linear regression model of log-transformed sRAGE

	B (95% C.I.)	Beta	*p*-value
Body mass index, g/m²	-5.67 (-9.64 ; -1.69)	-0.12	0.003
Insulin concentration, nU/ml	-3.79 (-5.92 ; -1.65)	-0.14	0.001
Creatinine clearance, µl/min	-1.39 (-2.13 ; -0.66)	-0.15	<0.001
Mycophenolate mofetil usage, yes/no	-0.08 (-0.12 ; -0.06)	-0.21	<0.001

Prospective follow-up was for 4.2 (3.7 – 4.6) years, with 72 (12%) deaths and 37 (6.1%) death censored graft losses at 2.5 (1.4 – 3.6) and 2.3 (0.9 – 3.2) years of follow-up respectively. Incidences and hazard ratios of crude and multivariate Cox-regression analyses for quartiles of sRAGE are shown in table 3. Mortality was significantly predicted by baseline sRAGE concentrations in renal transplant recipients, with low concentrations being a risk factor, and higher concentrations being protective. There was a significant ($p = 0.04$ for a quadratic term of sRAGE in a Cox-regression analyses with sRAGE as a continuous variable) curvilinear relationship between sRAGE and mortality, with the lowest hazard ratio for the second quartile of sRAGE (0.39 (0.19 – 0.79)), and higher hazard ratios for the third and fourth quartiles (0.54 (0.29 – 1.03) and 0.74 (0.41 – 1.31), respectively). Adjustment for donor age, recipient age, donor sex and recipient sex did not materially affect these associations (Model A). However, after adjustment for creatinine clearance, the hazard ratios of the upper two quartiles decreased to significant values of 0.41 (0.21 – 0.98) and 0.45 (0.23 – 0.85) respectively (Model B) ($p = 0.005$ for a quadratic term of sRAGE with sRAGE as a continuous variable in a Cox-regression analysis if this model). Further adjustment in Models C and D

did not materially influence the association of sRAGE with mortality. Figure 1 shows a Kaplan-Meier curve for subjects in the lowest quartile of sRAGE concentrations versus subjects in the three higher quartiles of sRAGE concentrations.

Table 3: Analyses of mortality and death censored graft loss for sRAGE quartiles

	Quartiles of sRAGE				
	I	II	III	IV	p-value
Mortality					
Incidence, n (%)	26 (17.7%)	11 (7.4%)	15 (10.1%)	21 (14.2%)	
Cox regression models [h]					
Crude	1.00	0.39 (0.19 – 0.79)	0.54 (0.29 – 1.03)	0.74 (0.41 – 1.31)	0.04
A [a]	1.00	0.35 (0.17 – 0.71)	0.51 (0.27 – 0.97)	0.68 (0.38 – 1.23)	0.02
B [b]	1.00	0.34 (0.17 – 0.70)	0.44 (0.23 - 0.84)	0.46 (0.25 – 0.85)	0.006
C [c]	1.00	0.33 (0.16 – 0.68)	0.43 (0.22 – 0.83)	0.50 (0.26 – 0.95)	0.008
D [d]	1.00	0.36 (0.18 – 0.75)	0.43 (0.22 – 0.85)	0.51 (0.26 – 0.97)	0.015
Death-censored graft loss					
Incidence, n (%)	7 (4.8%)	9 (6.1%)	7 (4.7%)	13 (8.8%)	
Cox regression models [h]					
Crude	1.00	1.20 (0.45 – 3.21)	0.95 (0.33 – 2.71)	1.78 (0.71 – 4.46)	0.48
A [a]	1.00	1.32 (0.49 – 3.56)	0.96 (0.34 – 2.73)	1.84 (0.73 – 4.66)	0.45
B [b]	1.00	0.60 (0.21 – 1.71)	0.85 (0.30 – 2.45)	0.83 (0.32 – 2.15)	0.80
C [c]	1.00	0.64 (0.22 – 1.91)	1.00 (0.33 – 3.03)	1.05 (0.39 – 2.85)	0.76
D [d]	1.00	0.50 (0.16 – 1.56)	0.89 (0.29 – 2.68)	1.02 (0.38 – 2.72)	0.53

[a] *Model A: adjusted for sex donor, sex recipient, age donor, age recipient*

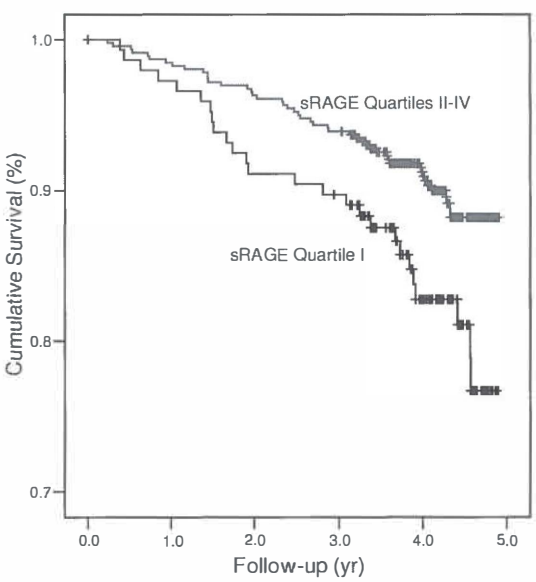
[b] *Model B: additionally adjusted for creatinine clearance*

[c] *Model C: additionally adjusted for BMI, fasting insulin and mycophenolate mofetil usage*

[d] *Model D: additionally adjusted for diabetes mellitus, history of MI, CRP and total cholesterol*

[h] *For Cox-regression models hazard ratios with 95% Confidence intervals (HR (95% CI)) are displayed*

Finally, we performed a multivariate analysis with continuous variables, from which it can be judged which factors were independent predictors of mortality in addition to sRAGE. In this model, sRAGE (β = -1.05 per ng/ml, p = 0.004), $sRAGE^2$ (β = 0.17 per ng^2/ml^2, p = 0.006), creatinine clearance (β = -0.38 per 10 ml/min, p < 0.001), age of the recipient (β = 0.65 per 10 years, p < 0.001), CRP (β = 0.42 per ^{10}log-transformed mg/l, p = 0.04), and diabetes mellitus (β = 0.75 per yes vs. no, p = 0.004) appeared to be independent predictors of mortality.



Baseline concentrations of sRAGE appeared not predictive of future death-censored graft loss, in neither the crude model, nor in the adjusted models.

Fig. 1: *Kaplan-Meier curve of patient survival for the lowest quartile of sRAGE concentration compared to the higher quartiles*

2.5 Discussion

To the best of our knowledge this is the first study on sRAGE in renal transplant recipients, and the second prospective study at large. We found use of mycophenolate mofetil, creatinine clearance, fasting insulin concentrations, and body mass index as significant independent determinants of plasma sRAGE concentrations in this population. We furthermore found that low levels of plasma sRAGE are independently associated with an increased risk of mortality in this population. Subjects with intermediate concentrations appeared to have the lowest risk.

Recent studies have shown that augmentation of atherosclerosis and development of nephropathy in diabetic mice can be inhibited by infusion of recombinant sRAGE, a c-terminal truncated secreted soluble isoform of the cell membrane receptor RAGE, which lacks the transmembrane and cytoplasmic domains(16;17). Consistent with a protective effect of sRAGE in humans, it has been demonstrated in a cross-sectional study that non-diabetic men with established coronary artery disease have significantly lower plasma sRAGE levels than men without coronary artery disease(23). We did not find an association between history of myocardial infarction and sRAGE concentrations in renal transplant recipients. Creatinine clearance has been identified as a more important determinant than such factors as prevalent coronary heart disease, hypertension or diabetes mellitus(24;25). We also found an inverse relation with creatinine clearance. The mechanism underlying the inverse association with creatinine clearance has been implicated glomerular filtration and subsequent tubular processing(24;25). It has remained a question to date whether up-regulation of sRAGE is a protective response against accumulation of AGEs and other toxins in patients with uremia.

We also found mycophenolate mofetil medication, BMI and fasting insulin concentrations as independent determinants of sRAGE. The association with use of mycophenolate mofetil as an immunosuppressive drug has not been reported before. The association with BMI and fasting insulin concentrations has not been reported before for sRAGE. However, consistent inverse associations have been reported for endogenous secretory RAGE (esRAGE)(20;26;27). esRAGE is one specific isoform of several different isoforms of proteins that are collectively referred to as sRAGE. Although sRAGE isoforms have slightly different protein sequences, they have in common that they contain the ligand-binding domain of the full-length RAGE protein, but not its transmembrane and signalling domains.

One of the studies reporting on esRAGE also contained a prospective part on the prediction of cardiovascular mortality in patients with end-stage renal disease(20). In this study, highest risk for cardiovascular mortality was present in patients with low esRAGE concentrations, and lowest risk in patients with intermediate concentrations. This is the same pattern which we find for the association of sRAGE with total mortality in renal transplant recipients. In contrast to our study, no association was found between esRAGE and renal function in the study in patients with end-stage renal disease(20). An explanation could be the much higher variation in renal function in our study population. We also performed a multivariate analysis from which it can be judged which variables predict mortality independently in addition to sRAGE. Creatinine clearance appeared one of these factors, in addition to age, CRP and diabetes.

How could high sRAGE concentrations, if not the consequence of poor renal function, be protective? In general, all kinds of sRAGE are hypothesized to have a RAGE-antagonizing role. This seems obvious since sRAGE proteins basically

comprise the extra-cellular domains of RAGE(28). But there is also evidence from studies, which employed murine models, that sRAGE possesses a RAGE-antagonizing effect(17-19). By these means sRAGE could be able to neutralize ligands of RAGE, such as advanced glycation endproducts (AGEs), which are known to have toxic effects in many morbidities(9;11). This hypothetical mechanism has been confirmed for diabetic nephropathy after ACE-inhibitor medication(29).

Our study has some limitations. One of them concerns sRAGE clearance, which was not measured in this study. Another limitation is the ELISA test for sRAGE detection, which we used. This test uses a non-epitope-tagged antibody, i.e. it cannot distinguish between several types of sRAGE. In the meantime another ELISA kit became commercially available, which is able to detect specifically esRAGE(30). A combination of both assays could have given a clearer picture of the differences between sRAGE and esRAGE. However, our results and the results of previous studies on esRAGE suggest great overlap in cross-sectional associations and predictive value(27). Third, this study measured sRAGE from plasma, which reflects a systemic picture rather than a local picture. We did not find an association between plasma sRAGE concentrations and subsequent graft loss. In order to see the effect of sRAGE on kidney function it might have been useful to measure sRAGE from kidney tissue directly. Fourth, there was only a relatively small number of death-censored graft losses, which lowers the power of detecting an association between death-censored graft loss and sRAGE. With a higher number it might have been possible to also detect this association. Last not least, it was not possible to split up mortality in cardio-vascular mortality and other causes. Given the similarity between our results of the prospective analyses with sRAGE and those of the prior prospective study of esRAGE in patients with end-stage renal disease(20), analyses restricted to cardiovascular mortality might have given an even stronger relationship between sRAGE and increase in risk.

In conclusion, we provide evidence that low levels of sRAGE levels are associated with increased risk for mortality from all causes, in particular if it is taken into account that high sRAGE concentrations can be the consequence of poor renal function. Given this association with increased mortality, our cross-sectional findings of an inverse association of sRAGE with use of mycophenolate mofetil, BMI and fasting insulin concentrations merits further investigation in the mechanisms underlying these associations, and whether use of mycophenolate mofetil indeed translates into a reduction in circulating concentrations of sRAGE.

2.6 Acknowledgements

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Chapter 3

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

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3.1 Abstract

BACKGROUND: 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.

BACKGROUND: 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.

3.2 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 transplantations 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 and 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 recognized 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.

3.3 Materials and Methods

3.3.1 Study design and patients

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.

3.3.2 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.

3.3.3 Skin-AF measurements

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%.

3.3.4 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 ischemic attack, or intermittent claudication in medical history of 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).

3.3.5 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.

3.3.6 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 analyzed 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)

3.3.7 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 table 1 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. A correlation matrix was constructed to detect potential collinearity between the baseline variables. Variables which had correlation coefficients higher than 0.8 were not included at the same time. Subsequently, variables that at least showed a trend ($P \leq 0.10$) 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. For this sub-analyses we applied univariable Cox-regression analyses only, since the number of end-points did not suffice to allow for multivariable models. 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)

3.4 Results

Table 1: Recipient and transplant characteristics

	Tertiles skin-AF			
	1.2-2.3 a.u. n=100	**2.3-2.9 a.u. n=101**	**2.9-5.2 a.u. n=101**	**P value**
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
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
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/m²)	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

Table 1 (continued)

	1.2-2.3 a.u.	2.3-2.9 a.u.	2.9-5.2 a.u.	P value
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
Transplant characteristics				
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
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(%).

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 table 1 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 (table 1).

Table 2: Results of univariable and multivariable Cox regression analysis

	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		

Table 2 (continued)

	Univariable		Multivariable	
	HR [95% C.I.]	**P value**	**HR [95% C.I.]**	**P value**
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		

Table 2 (continued)

	Univariable		Multivariable	
	HR [95% C.I.]	P value	HR [95% C.I.]	P value
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		

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 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 2. Skin-AF significantly predicted graft loss in a 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, 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 ischaemia 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 predictors of graft loss, including patient age, creatinine clearance, proteinuria, diabetes mellitus, HbA1c, and hsCRP were 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).

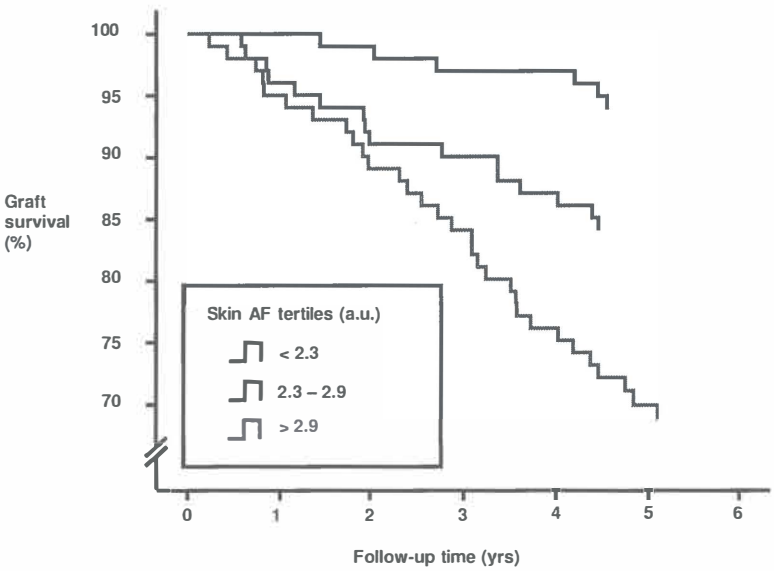

Fig. 1: Graft survival stratified for skin-AF tertile. Shown are unadjusted Cox-survival curves stratified on tertiles of skin-AF

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.

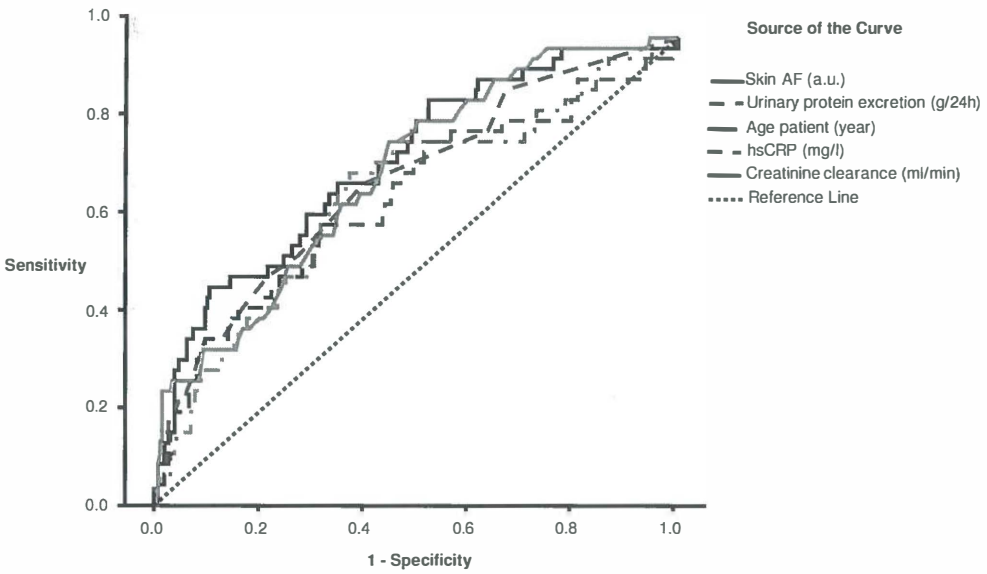

Fig. 2: *ROC curves for graft loss. Shown are the ROC curves of skin-AF, urinary protein excretion, age patient, hsCRP, and creatinine clearance.*

3.5 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 recognized 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 in stead of all-cause mortality and graft failure due to chronic transplant dysfunction in stead 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.

In the final model for graft loss some well-known predictors, like patient sex, use of calcineurin inhibitors, diabetes mellitus, HbA1c, glucose concentration, acute rejection, donor age, hypertension, hypercholesterolemia, BMI, and ischaemia times did not show predictive value. Some of these did show a (borderline) significant univariable relation with outcome. This suggests that a power issue may explain why we could not confirm these predictors.

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.

3.6 Acknowledgements

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Chapter 4

The N^{ε}-carboxymethyl-lysine (CML)-related risk for mortality in renal transplant recipients is modified by creatinine clearance

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4.1 Abstract

INTRODUCTION: Long-term outcome after renal transplantation is compromised by high rates of mortality, mainly due to cardiovascular disease (CVD) and graft failure, mainly due to chronic transplant dysfunction (CTD). Accumulation of advanced glycation end-products (AGEs) may contribute to both CVD and CTD. We investigated potential determinants of plasma levels of the AGE N^{ε}-carboxymethyl-lysine (CML) in renal transplant recipients and the association of CML with mortality and graft failure, both with focus on potential interactions with renal function and diabetes.

METHODS: Outpatient-clinic renal transplant recipients (RTR) with a graft functioning for >1 year were eligible for participation. CML was determined baseline from plasma using liquid chromatography and mass spectrometry (LC-MS/MS). Mortality and graft failure were recorded until September 2007. Multivariable linear regression analyses were performed to identify potential determinants of CML. Multivariable Cox-regression analyses including interaction of CML and CCL as well as interaction of CML and diabetes were performed to investigate whether CML could predict mortality or graft failure.

RESULTS: 555 RTR participated in this study. Median CML concentration was 1.8 (1.5–2.1) µmol/l. CML levels were independently associated with BMI (beta=-0.12, p=0.003), serum albumin (beta=0.16, p<0.001), and CCL (beta=-0.37, p<0.001). Prospectively, low CML was an independent risk factor for mortality, but there was also a significant interaction with CLL, with an increased risk for mortality in RTR with both high CML and low CCL. CML was not independently predictive of graft failure. No significant interaction was found between CML and diabetes for prediction of mortality or graft failure.

CONCLUSIONS: Plasma levels of CML in RTR may mainly be determined by kidney function but not or mildly be determined by diabetes. In contrast to expectations, low plasma CML is an independent risk factor for mortality. High levels of CML are a risk factor for mortality in RTR with low kidney function, possibly as a marker for a truly low renal function or a high metabolic rate.

4.2 Introduction

Kidney transplantation is the preferred treatment for patients with end-stage renal disease, both in terms of quality of life and patient survival (1;2). Long-term outcomes of kidney transplantation remain, however, relatively disappointing, with age-adjusted mortality rates approximately 3-5 times higher than in the general population (3) and loss of graft function in > 50% of cases at 10 year after transplantation (4;5).

Advanced glycation end-products (AGEs) are believed to compromise both patient survival and graft survival. Evidence has been reviewed for a central role of AGEs in the development of cardiovascular disease and chronic transplant dysfunction, which are the most common reasons for mortality and graft failure after kidney transplantation (6;7). AGEs are a heterogeneous group of irreversibly modified molecules, amongst which N^{ε}-carboxymethyl-lysine (CML) is one of the most abundant in humans (8). In general AGEs are believed to have toxic effects either by eliciting pro-inflammatory signaling via AGE-receptors or by cross-linking endogenous molecules (9;10). It has been shown that in healthy subjects, CML is cleared efficiently by the kidney, and that this probably prevents damaging effects of exposure to CML (11). In patients with poor kidney function CML may accumulate as a consequence of decreased clearance while in patients with diabetes it may accumulate as a consequence of increased production in the

context of hyperglycemia (12). Both renal function and diabetes could therefore be modifiers of a putatively increased risk for mortality and graft failure associated with CML.

In the present study we measured plasma CML levels in renal transplant recipients using liquid chromatography combined with mass spectrometry (LC-MS/MS). In cross-sectional analyses, we explored potential determinants of CML and further, we prospectively investigated whether CML could predict patient mortality and graft failure, taking into account potential interaction with CCL and diabetes.

4.3 Materials & Methods

4.3.1 Study design and patients

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 in accordance with American Transplantation Guidelines (13) in the outpatient clinic, i.e. ranging from twice a week just after discharge from hospital to twice a year long-term after transplantation. Between December 2001 and July 2003, all adult allograft recipients who survived the first year after transplantation with a functioning allograft were eligible to participate in this study at their next visit to the outpatient clinic. A total of 606 renal transplant recipients signed written informed consent, from an eligible 847 (72% consent rate). The group that did not sign informed consent was comparable with the group that signed informed consent with respect to age, sex, body mass index, plasma creatinine, CCL, and proteinuria. For 51 patients no plasma samples were available leaving 555 patients included for our analyses. Follow-up time was defined as the time from inclusion into the study until death, or the first occurrence of graft failure. Graft failure was

defined as return to dialysis therapy or need for re-transplantation. Cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. Causes of death were coded according to the International Classification of Diseases, 9th revision (ICD-9). There was no loss to follow up. Up-to-date information on patient status was ensured by our outpatient program, which operates in close collaboration with referral hospitals in our area.

4.3.2 Recipient and transplant characteristics

Relevant donor, recipient and transplant characteristics were extracted from the Groningen Renal Transplant Database. This database holds information of all renal transplantations that have been performed at our center since 1968. Extracted were donor and recipient age and sex, duration of pre-transplant dialysis, date of transplantation, transplantation type, ischemia times, HLA mismatches, renal function at baseline, type of acute rejection treatment, and use of medication. From a self-report questionnaire we received details about history of cardiovascular disease and smoking. Smoking was defined as current use of cigarettes. Current medication was extracted from medical records. Patients were defined as having experienced an episode of acute rejection, when drugs were used to treat acute rejection. Standard immunosuppressive therapy consisted of the following: from 1968 until 1989, prednisolone and azathioprine (100 mg/day; from January 1989 to February 1993, cyclosporine standard formulation (Sandimmune, Novartis; 10 mg/kg; through levels of 175-200 µg/l in first 3 months, 150 µg/l between 3 and 12 months post-transplant, and 100 µg/l thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day). From March 1993 to May 1996, cyclosporine micro-emulsion (Neural; Novartis Pharma B.V., Arnhem, The Netherlands; 10 mg/kg; trough levels idem) and prednisolone and from May 1996 to date, mycophenolate mofetil (Cellcept; Roche b.v., Woerden, The Netherlands; 2 g/day) was added.

4.3.3 Clinical measurements

During the visit to the outpatient clinic, blood pressure was measured using an automated oscillometric blood pressure device (Omron M4, Omron Europe BV, the Netherlands) as the average of 3 consecutive measurements with 1-minute intervals after a 6 minutes rest in supine position. Height and weight were assessed as well during this visit and the body mass index (BMI) was calculated. Waist circumference was measured on bare skin midway between the 10th rib and the iliac crest, hip circumference was measured at the maximum circumference of the buttocks, and a waist to hip ratio was calculated from these measurements.

4.3.4 Laboratory measurements

All measurements, including blood sampling were performed after an 8-12h overnight fasting period for food and medication. 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 analyzed for concentrations of glucose, triglycerides and total cholesterol using standard laboratory techniques. Low density lipoprotein (LDL) was calculated using the Friedewald formula. 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 (hsCRP) ELISA assay. Both intra- and inter-assay variation coefficients were 5%. Procalcitonin analyses were performed using an ultrasensitive immunoluminometric assay (BRAHMS PCT sensitive LIA; BRAHMS Aktiengesellschaft, Hennigsdorf, Germany) (14). Plasma soluble intercellular adhesion molecule type 1 (sICAM-1) and plasma soluble vascular cellular adhesion molecule type 1 (sVCAM-1) concentrations were measured as markers of ED by ELISA kits (Diaclone Research, Besancon, France) (15). Creatinine clearance (CCL) was calculated from 24 hour urine by the UxV/P formula. Serum albumin was determined with a

Roche Modular P (Roche diagnostics GmbH, Mannheim, Germany) by use of a bromcresol green dye-binding method. 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 (16).

4.3.5 CML measurement by LC-MS/MS

CML was determined by separation of plasma components on a liquid chromatograph (LC) and subsequent analysis of the protein fraction in a stable-isotope-dilution tandem mass spectrometer (MS/MS) as it was described previously (17). In short, CML was liberated from plasma proteins by acidic hydrolysis after addition of deuterated CML as internal standard. 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 for CML, and 209.1 → 388.1 for its respective internal standard were monitored in positive-ion mode. CML was separated with baseline resolution with a total analysis time of 21 min. Within-day and between-day coefficients of variation were <4.4% and <3.2%.

4.3.6 Statistical analyses

Analyses were performed with SPSS version 16 (SPSS Inc., Chicago, IL, USA). Variables showing normal distribution are expressed as mean ± standard deviation, whereas variables with a skewed distribution are given as median (interquartile range) and nominal variables are expressed as n (%). A p-value of 0.05 or smaller was considered to indicate statistical significance.

Baseline data were cross-sectionally investigated for associations over quintiles of CML applying one-way ANOVA for parametric variables, Jonckheere-Terpstra test for skewed variables and Pearson chi-square test for nominal variables. Variables which showed a significant association with CML were further analyzed in subsequent univariable and multivariable backward linear regression analyses with CML as dependent variable. For these analyses, the distribution of CML was normalized by log-transformation.

The predictive value of CML levels for the risk of mortality or graft failure was analyzed using univariable and multivariable Cox proportional hazard models. For the initial univariate models CML was included as CML-quintiles. For later models, which investigated a potential interaction of CML with CCL and diabetes for prediction of outcome, CML was included as continuous variable. The multivariable Cox-models included donor age and sex, recipient age and sex and the potential determinants of CML found from the cross-sectional analyses.

Table 1a: *Patient characteristics*

	CML quintiles (µM)					
	1 (0.88-1.39)	2 (1.39-1.63)	3 (1.64-1.87)	4 (1.90-2.15)	5 (2.16-5.25)	P
Demographics						
Age pat. (yr)	49.6 ± 13.2	51.6 ± 12.6	51.4 ± 11.5	52.6 ± 12	51.7 ± 11.6	0.5
Male pat., n (%)	58 (52.7)	67 (59.8)	65 (58.6)	60 (54.1)	60 (54.1)	0.8
Body composition						
BMI (kg/m²)	27.4 ± 4.9	26.5 ± 3.7	25.4 ± 4.7	25.2 ± 3.6	25.5 ± 4.2	<0.001
Waist-hip ratio	0.99 ± 0.10	1.00 ± 0.10	0.96 ± 0.11	0.96 ± 0.10	0.97 ± 0.11	0.02
PTWG (kg)*	3.5 ± 8.0	2.5 ± 6.8	1.4 ± 6.2	2.2 ± 6.2	1.0 ± 6.7	0.07
Blood pressure						
SBP (mmHg)	151.6 ± 21.2	151.7 ± 22	149.9 ± 20.7	157.3 ± 23.2	155.7 ± 24.8	0.08
DBP (mmHg)	90.9 ± 9.9	89.6 ± 9.6	89.5 ± 9.4	90.8 ± 9.3	89.3 ± 10.2	0.6
Antihyp., n (%)	96 (87.3)	97 (86.6)	94 (84.7)	98 (88.3)	100 (90.1)	0.8
ACE inh., n (%)	39 (35.5)	35 (31.3)	36 (32.4)	33 (29.7)	47 (42.3)	0.3
Beta-bl., n (%)	72 (65.5)	67 (59.8)	67 (60.4)	69 (62.2)	69 (62.2)	0.9
Calc.ant., n (%)	40 (36.4)	41 (36.6)	39 (35.1)	49 (44.1)	43 (38.7)	0.7

Table 1a continued	1	2	3	4	5	P
			Cardiovascular risk factors			
TIA/CVA, n (%)	5 (4.7)	5 (4.5)	8 (7.2)	9 (8.1)	6 (5.5)	0.7
Smoking (current), n (%)	31 (28.2)	26 (23.2)	26 (23.4)	19 (17.1)	20 (18)	0.3
Total chol. (mmol/l)	5.6 ± 1.0	5.5 ± 0.9	5.7 ± 1.0	5.8 ± 1.3	5.6 ± 1.0	0.2
LDL chol. (mmol/l)	3.4 ± 0.9	3.4 ± 0.9	3.6 ± 1.0	3.7 ± 1.1	3.5 ± 1.0	0.06
Statine, n (%)	54 (49.1)	56 (50)	47 (42.3)	59 (53.2)	57 (51.4)	0.6
Triglycerides (mmol/l)	1.9 [1.4-2.7]	2 [1.4-2.5]	1.9 [1.4-2.7]	1.8 [1.4-2.7]	2 [1.5-2.9]	0.5
Diabetes						
Diabetes mellitus, n (%)	22 (20)	18 (16.1)	19 (17.1)	16 (14.4)	21 (18.9)	0.8
Antidiabetica usage, n (%)	16 (14.5)	15 (13.4)	14 (12.6)	12 (10.8)	17 (15.3)	0.9
Glucose conc. (mmol/l)	4.6 [4.3-5]	4.6 [4.1-5]	4.5 [4-5.1]	4.4 [4-4.9]	4.6 [4.1-5]	0.2
Insulin conc. (µU/ml)	11.6 [8.7-17.5]	11.7 [8.9-18.1]	10.9 [7.6-16.3]	10.8 [7.8-14.7]	10.3 [7.4-13.7]	0.004
HbA1c (%)	6.4 ± 1.0	6.4 ± 1.0	6.5 ± 1.1	6.5 ± 1.1	6.7 ± 1.1	0.3
History of kidney failure						
Previous dialysis, n (%)	6 (5.5)	11 (9.8)	8 (7.2)	6 (5.4)	16 (14.4)	0.09
Previous transpl., n (%)	10 (9.1)	12 (10.7)	14 (12.6)	10 (9)	13 (11.7)	0.9
Inflammation						
Albumine conc. (mg/ml)	39.9 ± 3.6	40.4 ± 3.9	40.9 ± 2.9	40.9 ± 3.0	40.9 ± 3.4	0.1
hsCRP con. (µg/ml)	2.5 [1-6.7]	2.6 [1-5.3]	1.6 [0.7-4.1]	1.7 [0.7-4.8]	1.8 [0.6-5.1]	0.02
sRAGE (ng/ml)	1.46 [1.12-2.05]	1.73 [1.10-2.25]	1.60 [1.29-2.10]	1.76 [1.31-2.38]	1.79 [1.46-2.57]	<0.001
sVCAM-1 (ng/ml)	0.96 [0.78-1.17]	0.91 [0.76-1.21]	1.03 [0.77-1.22]	0.91 [0.74-1.18]	1.05 [0.81-1.27]	0.2
sICAM-1 (ng/ml)	0.60 [0.50-0.70]	0.62 [0.53-0.76]	0.61 [0.52-0.75]	0.59 [0.51-0.71]	0.60 [0.52-0.77]	0.7
PCT (µg/ml)*	23.0 [16.0-32.0]	23.0 [17.0-32.0]	21.0 [18.0-31.0]	22.0 [17.0-35.0]	28.0 [20.0-45.0]	0.01

*Note: PTWG = post transplant weight gain, PCT = Procalcitonin

4.4 Results

A total of 555 outpatients (age 52 ± 12 years, 55% males, CCL 62 ± 22 ml/min) participated at a median of 6.0 (2.7 – 11.4) years after transplantation in the baseline measurements of our study. Median follow-up time was 5.3 (4.7-5.7) years. Plasma concentrations of CML were 1.8 (1.5 – 2.1) µmol/l.

The recipient and donor characteristics stratified for CML quintiles are shown in table 1a and 1b, respectively. CML had significant associations with BMI ($p<0.001$), insulin concentrations ($p=0.004$), serum albumin ($p=0.04$), hsCRP ($p=0.02$), soluble receptor for AGEs (sRAGE) ($p<0.001$), procalcitonin ($p=0.01$), donor age ($p=0.03$), donor sex ($p=0.01$), CCL ($p<0.001$), living donor transplantation ($p=0.03$) and usage of proliferation inhibitors ($p=0.04$). Trends were shown by SBP ($p=0.08$), LDL cholesterol ($p=0.06$) and previous dialysis ($p=0.09$). In order to identify independent associations, these variables were analysed in univariable and multivariable linear regression models.

Insulin levels showed a negative trend (beta=-0.11, $p=0.01$), which, became insignificant after adding BMI to the model (beta=-0.06, $p=0.19$). SBP showed a positive trend (beta=0.11, $p=0.007$), which became stronger after adding BMI to the model (beta=0.14, $p=0.001$). However, after also adding CCL to this model, the significant association of SBP with CML was lost (beta=0.06, $p=0.1$). Besides SBP also the trends of donor age (beta=0.02, $p=0.5$), donor sex (beta=-0.07, $p=0.09$) and proliferation inhibitor usage (beta=-0.07, $p=0.06$) became insignificant after adding CCL to the respective model. Serum levels of hsCRP showed a significant trend with CML after logarithmic transformation (beta=-0.08, $p=0.05$). This trend, however, became insignificant after adding serum albumin to the model (beta=-0.07, $p=0.13$). BMI (beta=-0.16, $p<0.001$), serum albumin (beta=0.09, $p=0.04$) and

CCL (beta=-0.39, p<0.001) showed significant associations with CML. Log-transformed procalcitonin (beta=0.15, p=0.001) and sRAGE (beta=0.16, p<0.001) showed positive associations with CML, which both became insignificant after adding CCL to the model.

Table 1b: *Donor/graft characteristics*

	CML quintiles					
	I	II	III	IV	V	P
CML range (µM)	**(0.88-1.39)**	**(1.39-1.63)**	**(1.64-1.87)**	**(1.90-2.15)**	**(2.16-5.25)**	
Demographics						
Age donor (yr)	34.8 ± 15.1	36.1 ± 14.7	36.5 ± 14.4	35.5 ± 16.6	40.9 ± 15.4	0.03
Male, n (%)	65 (59.1)	74 (66.1)	49 (44.1)	64 (58.2)	55 (50)	0.01
Allograft viability						
Warm isch. (min)	35 [30-44]	35 [30-45]	37 [30-46]	35 [30-43]	35 [30-45]	0.9
Cold isch. (h)	21 [9-28]	22 [14-26]	22 [15-27]	23 [17-28]	20 [14-27]	0.4
Living don., n (%)	22 (20%)	16 (14.3)	16 (14.4)	6 (5.4)	18 (16.2)	0.03
HLA AB mism., n (%)	81 (73.6)	84 (75)	79 (71.2)	80 (72.1)	77 (69.4)	0.9
HLA DR mism., n (%)	40 (37.7)	45 (40.5)	41 (38.3)	38 (34.2)	40 (36.7)	0.9
Time ntx → baseline (yrs)	5.0 [2.2-10.3]	6.1 [3.2-12.3]	7.1 [2.6-12.8]	6.6 [3.5-10.7]	5.8 [2.5-10.6]	0.7
Allograft function						
CCL (ml/min)	72.2 ± 22.8	67.1 ± 20.3	61.5 ± 18.8	61.1 ± 21.4	46.6 ± 18.9	<0.001
Creatinine con. (µmol/l)	119 [98-143]	125 [109-146]	133 [116-155]	139 [116-167]	171 [137-226]	<0.001
Urinary protein excr. (g/24h)	0.2 [0.1-0.6]	0.2 [0-0.4]	0.2 [0-0.5]	0.2 [0-0.4]	0.3 [0.2-0.6]	0.4
Acute rejection, n (%)	42 (38.2)	57 (50.9)	47 (42.3)	47 (42.3)	60 (54.1)	0.1
Immunosuppression medication						
Prednisolon dose (mg/day)	10 [7.5-10]	10 [7.5-10]	10 [8.8-10]	10 [7.5-10]	10 [8.8-10]	0.7
Calcineurine inh., n (%)	82 (74.5)	86 (76.8)	86 (77.5)	99 (89.2)	85 (76.6)	0.06
Proliferation inh., n (%)	88 (80)	88 (78.6)	84 (75.7)	71 (64.0)	78 (70.3)	0.04

The result of the backward linear regression analysis is shown in table 2. From this analysis it appeared that BMI, serum albumin and CCL were the only variables which stayed significant with inclusion of other variables and were therefore considered as potential determinants of CML. BMI (beta=-0.11, p=0.007) showed a slight decrease in beta and p-value compared to the univariate model. Serum albumine (beta=0.14, p=0.001) on the contrary showed an increase in beta and p-value compared to the univariable model, which was due to addition of CCL to the model. CCL (beta=-0.40, p<0.001) only showed a marginal difference compared to the univariable model.

Table 2: *Multivariable backward linear regression of log-transformed CML*

	Univariable			Multivariable [a]		
	B	**Beta**	**P**	**B**	**Beta**	**P**
BMI (kg/m²)	-0.005	-0.16	<0.001	-0.003	-0.12	0.003
SBP (mmHg)	0.001	0.11	0.007			
LDL cholesterol (mmol/l)	0.006	0.05	0.2			
Insulin (µU/ml)	-0.002	-0.11	0.01			
Previous dialysis	0.026	0.06	0.2			
Albumine (g/l)	0.003	0.09	0.04	0.006	0.16	<0.001
hsCRP (log (mg/ml))	-0.017	-0.08	0.05			
sRAGE (ng/ml)	0.018	0.16	<0.001			
PCT (log(ng/ml))	0.066	0.15	0.001			
Age donor (yr)	0.001	0.12	0.006			
Sex donor (male)	-0.020	-0.08	0.05			
Living Donor (yes)	-0.021	-0.06	0.2			
CCL (ml/min)	-0.002	-0.39	<0.001	-0.002	-0.37	<0.001
Proliferation inhibitor usage	-0.025	-0.09	0.03			

[a] *R^2 of the multivariable model = 0.18*

We visualized the relation of CML and CCL in a scatter plot (fig. 1). From curve estimation analyses is occurred that the logarithmic relation had the highest probability to be true.

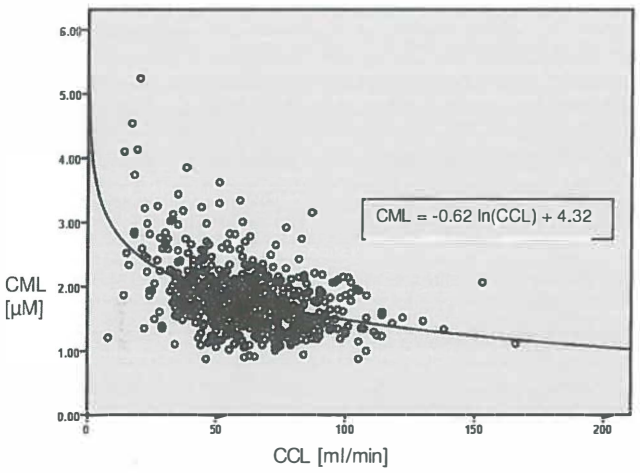

Fig. 1: *Shown is the scatter plot of CML with CCL. The line indicates the best-fit line, which is explained by the formula in the box.*

The results of Cox-regression analyses for mortality according to quintiles of CML are shown in table 3. These analyses show that there is a U-shaped association of CML with mortality. Adjustment for potential confounders in subsequent additive models did not materially affect this association.

There was no significant association of CML with future development of graft failure.

In order to evaluate a potential interaction between CML and CCL and diabetes for the risk of mortality and graft failure, multivariable Cox-models including CML (as continuous variable), CCL/diabetes and a product-term of CML and CCL/diabetes were established (table 4). These models showed that there was a significant interaction between CML and CCL for the prediction of mortality (p=0.002). No significant interaction was found for diabetes.

Table 3: Prospective analyses

	CML quintiles				
	I	II	III	IV	V
N (%)	20 (18.2%)	13 (11.6%)	9 (8.1%)	17 (15.3%)	26 (23.4%)
crude	2.39 (1.07-5.25)*	1.40 (0.60-3.29)	1 (reference)	1.95 (0.87-4.39)	3.29 (1.54-7.02)**
A	2.40 (1.08-5.30)*	1.20 (0.51-2.83)	1 (reference)	1.70 (0.75-3.83)	3.26 (1.52-6.99)**
B	2.94 (1.32-6.52)**	1.29 (0.55-3.04)	1 (reference)	1.68 (0.75-3.78)	2.57 (1.19-5.55)*
C	2.59 (1.16-5.78)*	1.09 (0.45-2.62)	1 (reference)	1.65 (0.73-3.71)	2.69 (1.24-5.82)**
D	2.62 (1.18-5.83)*	1.13 (0.47-2.72)	1 (reference)	1.76 (0.78-4.01)	2.76 (1.27-6.00)**
E	2.65 (1.19-5.91)*	1.06 (0.44-2.55)	1 (reference)	1.72 (0.76-3.88)	2.58 (1.19-5.61)*

Model A: Adjustment for sex donor/recipient, age donor/recipient

Model B: Model A, with additional adjustment for CCL

Model C: Model B, further adjusted for albumin

Model D: Model C, further adjusted for BMI

Model E: Model D, further adjusted for diabetes

** p≤0.05, **p≤0.01*

In order to visualize this interaction between CML and CCL for prediction of mortality, three-dimensional plots were calculated showing the incidences of mortality over CML tertiles and high/low CCL (Fig. 2). In this plot a clear tendency for higher mortality with lower CML could be seen. Furthermore, the association of high CML with increased risk for mortality is only present in RTR with low CCL.

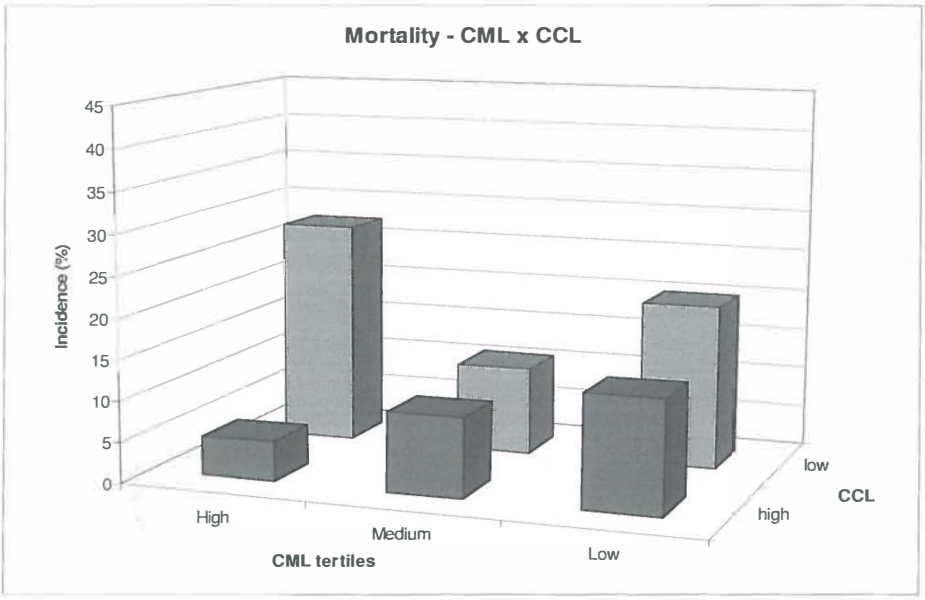

Fig. 2: Shown are the incidences of mortality over CML tertiles and CCL lower / higher than the mean.

Table 4: Multivariate Cox-regression for mortality corrected for interaction [a]

	B	Wald	P	HR (95% CI)
Creatinine clearance				
CML	1.13	16.1	<0.001	3.10 (1.78-5.40)
CCL	0.02	2.9	0.09	1.02 (1.00-1.05)
CML* CCL	-0.03	12.4	<0.001	0.97 (0.96-0.99)
Diabetes				
CML	0.19	0.14	0.7	1.21 (0.44-3.38)
Diabetes	-0.09	0.02	0.9	0.92 (.023-3.61)
CML * diabetes	0.16	0.23	0.6	1.17 (0.62-2.22)

[a] *also corrected for donor/recipient sex, donor/recipient age, BMI and albumin*

4.5 Discussion

To the best of our knowledge, this is the first study which examined plasma CML levels in kidney transplant recipients. In our cross-sectional analyses we found BMI, albumin and CCL to be independently associated with CML levels. In our prospective studies we found that the CML-related risk for mortality is modified by CCL in such a way that high CML levels are a risk factor for mortality if CCL is low, and low CML levels are a risk factor for mortality if CCL is high. No association of CML with diabetes was observed.

In our study, we found an independent inverse association of BMI with CML. Literature reporting on CML is mainly in diabetes, since CML and other AGEs are classically known to accumulate as a consequence of increased production (18). Since diabetes type II and obesity are correlated, one might anticipate a positive association of CML and BMI. However, the prevalence of diabetes in our population was 17.8%, and diabetes was not independently related to CML in our population. The inverse association of BMI with CML therefore results from other factors than diabetes. An inverse association of obesity with serum glycated albumin and CML has also been observed in obese children and adults, and attributed to a chronic inflammation-induced increased albumin turnover (19-21). We also measured total CML, which is mainly protein-bound, so the same phenomenon may explain our finding of an inverse association. In our study, serum albumin was positively related to CML, which is consistent with albumin being the main plasma protein carrying CML. We also found an inverse association of CML with renal function, which is consistent with kidney playing an important role in clearance of AGEs, but it could also be consistent with the notion that AGEs deteriorate kidney function. For both hypotheses large bodies of evidence exist in literature (6;11). Considering both principles together, a negative feedback-cycle could exist by which AGEs deteriorate kidney function, which then leads to

accumulation of AGEs, which further deteriorates kidney function and so forth. In our prospective analyses, however, we did not find an association of CML with development of graft failure, which may be considered an argument against a detrimental effect of AGEs on renal function itself.

In our prospective analyses concerning a putative association of CML with mortality, we found high CML to only be associated with increased mortality in the circumstance of low CCL. If CCL was high, high CML was protective. Our study is the first to report on CML as a risk factor for mortality in renal transplant recipients. In two studies in haemodialysis patients, it was reported that high levels of AGEs are associated with increased mortality (22-24) . In these studies the results were not corrected for AGE-removal. In another study, however, the results were corrected for the usage of a high-flux membrane, which may be seen as an analogous measure for kidney function (28). Interestingly, the results for the prediction of mortality in this study showed a similar shape as in our study when interaction with CCL was not taken into account. The third CML quartile in the study of Schwedler *et al.* was the one with the lowest hazard ratio for all-cause mortality, while the first quartile had the highest hazard ratio. In our study we, analogously, found that the first and the fifth quintile had the highest hazard ratio, while the third quintile had the lowest. Apparently, high CML in the context of poor renal function is an indicator of true renal function insufficiency, with renal function being too poor to allow for sustenance of life, while high CML in the context of good renal function is an indicator of good health.

Several pathways have been hypothesized by which AGEs could cause mortality in patients with decreased kidney function. Some studies investigated the fate of endogenous AGEs and early studies showed that the kidney is involved in the removal of AGEs on the example of the catabolism of pentosidine (25;26). Although CML does not seem to be catabolized in the kidney, it has been shown

that it is still removed efficiently by the kidney. Rat experiments showed that intravenously injected CML is excreted by the kidney to approximately 87% within two hours (27). The authors of this study suggest that under healthy conditions AGEs are cleared too quickly to cause serious damage to the organism. In uraemia, however, AGEs may accumulate to such an extend that they can cause serious damage. The pathology of AGEs was investigated widely for diabetic complications and other chronic disorders (28). AGEs exert their toxic effect partly by changing the physico-chemical characteristics of proteins and partly by binding to a transmembrane receptor (RAGE) which activates pro-inflammatory signalling cascades (12). The transcription factor NFκB thereby probably plays a central role by prolonging the pro-inflammatory signal, which in the end may lead to chronic disorders (29).

In our analyses we neither found a significant association of diabetes with CML nor an interaction of CML and diabetes in predicting mortality. Further, a negative association of insulin with CML was found, opposite to an expected positive relation. This association, however, depended on BMI. Together these findings indicate that diabetes in our population plays a minor or even no role in determining CML levels.

In summary, our results show that plasma total CML is inversely associated with BMI and renal function and positively with albumin concentrations. CML is not associated with graft failure and high levels of CML are associated with increased mortality in subjects with poor renal function. In renal transplant recipients with good renal function, CML is inversely associated with mortality. Currently, no hypotheses exist on how high levels of CML could protect against mortaltiy, but results of other studies indicating inverse associations of CML with BMI and increased CML and protection against mortality in hemodialysis patients indicate that our finding of a counterintuitive association is not on its own. Therefore, further

research is needed to resolve, whether this is an epiphenomenon or whether high CML under certain circumstances could really be protective.

4.6 Acknowledgements

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Chapter 5

Body mass index and creatinine clearance are associated with steady state serum concentrations of the cell damage marker S100B in renal transplant recipients

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5.1 Abstract

BACKGROUND: S100B is a prominent cell damage marker, which can lead to sustained pro-inflammatory signaling. We aimed to investigate cross-sectional associations of steady-state S100B concentrations, particularly with C-reactive protein (CRP) in renal transplant recipients (RTR). Furthermore, we aimed to prospectively investigate whether S100B would predict graft failure or mortality.

MATERIALS & METHODS: Outpatient-clinic RTR with a graft functioning for >1 year were eligible for participation in this study. S100B was determined baseline from serum. Mortality and occurrence of graft failure were recorded until September 2007. Multivariable linear regression analyses were performed to identify potential determinants of S100B. Multivariable Cox-regression analyses were performed to investigate S100B as a potential predictor of mortality or graft failure.

RESULTS: 581 renal transplant recipients participated in this study. Median S100B concentration was 0.19 [0.14 – 0.25] µg/L. Recipient age (beta=0.009, p=0.02), body mass index (BMI) (beta=0.021, p<0.001) and creatinine clearance (beta=-015, p<0.001) were independently associated with S100B. During follow-up, n=95 (16.4%) died and n=41 (7.1%) developed graft failure. S100B levels did not predict mortality or graft failure.

CONCLUSION: BMI, creatinine clearance, and age are determinants of steady-state serum S100B concentrations in renal transplant recipients. The association of BMI with S100B suggests that S100B might be a new adipokine.

5.2 Introduction

Renal transplantation is the preferred treatment for patients with end-stage renal disease, both in terms of patient survival and quality of life(1;2). One-year graft survival after renal transplantation has improved impressively over the past decades. Long-term allograft survival has, however, not paralleled this improvement(3;4). One main reason for this poor long-term outcome is premature death (with a functioning graft) because of cardiovascular disease (CVD)(5;6). Another reason is the gradual decline in graft function owing to chronic transplant dysfunction, ultimately requiring return to dialysis or re-tranplantation(7;8). The risk for both graft failure and mortality is related to chronic low-grade inflammation(9;10).

S100B is a protein of the S100 family which is involved in pro-inflammatory signaling through the receptor for advanced glycation end-products (RAGE)(11). S100B is primarily found in neuronal tissue and has been proposed as neuropathologic biomarker with a similar impact as c-reactive protein (CRP)(12). Elevated S100B levels were also found in patients with active systemic lupus erythematosus (SLE) without involvement of the central nervous system(13). This indicates a putative role for S100B in causing tissue damage and inflammation independent of neuronal injury. S100B may sustain inflammatory signaling by activating RAGE(11). This receptor has been implicated in having a central role in diabetic complications, due to pro-inflammatory signaling upon binding of advanced glycation end-products(14). More recently, RAGE was also implicated in having a central role in uraemia and dialysis by perpetuating the inflammatory response(15).

In this study we aimed to investigate, firstly, whether S100B is associated with inflammation (measured as CRP levels) in renal transplant recipients with graft

survival beyond one year after transplantation. Secondly, we investigated whether there were other factors, which could explain S100B levels in these patients. Thirdly, we prospectively investigated whether S100B could predict graft failure or mortality in these patients.

5.3 Materials & Methods

5.3.1 Study design and patients

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 in accordance with American Transplantation Guidelines(16) in the outpatient clinic, i.e. ranging from twice weekly just after discharge from hospital to twice yearly long-term after transplantation. 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 between December 2001 and July 2003. A total of 606 out of 847 eligible renal transplant recipients signed written informed consent. Samples for determination of S100B were available in 581 patients. All measurements, including blood sampling were performed after an 8-12h overnight fasting period of food and medication. Patients were followed-up for the first occurrence of graft loss. Graft loss was defined as re-admission to dialysis, re-transplantation, or patient death. Our outpatient program, which operates in close collaboration with referral hospitals in our area, ensured up-to-date information on patient status.

5.3.2 Recipient and transplant characteristics

Relevant donor, recipient, and transplant characteristics were extracted from the

Groningen Renal Transplant Database. This database holds information on all renal transplantations that have been performed at our center since 1968. Data extracted were on donor and recipient age and sex, duration of pre-transplant dialysis, date of transplantation, transplantation type, ischemia time, HLA mismatches, renal function at baseline, type of acute rejection treatment, and use of medication. We received details on history of cardiovascular disease and smoking from a self-report questionnaire. History of CVD was defined as having had at least one cerebrovascular accident or transient ischemic attack in the medical history of the patient. Smoking was defined as current use of cigarettes. Current medication was extracted from medical records. Patients were defined as having experienced an episode of rejection, when drugs were used to treat rejection. Patients were treated with standard immunosuppressive therapy as described before(17).

5.3.3 Clinical measurements

Blood pressure was measured using an automated oscillometric blood pressure device (Omron M4, Omron Europe BV, the Netherlands) during the visit to the outpatient clinic. Height and weight were also assessed during this visit and the body mass index (BMI) was calculated. Waist circumference was measured on bare skin midway between the 10th rib and the iliac crest.

5.3.4 Laboratory measurements

Blood was drawn at the outpatient clinic and 24-hour urine samples were collected. S100B was measured using a commercially available ELISA kit (Sangtech®-100 by DiaSorin S.p.A., Strada per Crescentino I, 13040 Saluggia (Vercelli), Italy). This test detects the beta-subunit of S100 proteins, and therefore measures S100ββ and S100αβ dimers in serum(18). We assessed the urine for concentrations of protein and creatinine, and analyzed the blood 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(19). Procalcitonin analyses were performed using an ultrasensitive immunoluminometric assay (BRAHMS PCT sensitive LIA; BRAHMS Aktiengesellschaft, Hennigsdorf, Germany)(20). Plasma soluble intercellular adhesion molecule type 1 (sICAM-1) and plasma soluble vascular cellular adhesion molecule type 1 (sVCAM-1) concentrations were measured as markers of ED by ELISA kits (Diaclone Research, Besancon, France)(21).

Creatinine clearance was calculated from 24-hour urine by the UxV/P formula. We classified diabetes mellitus 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(22). IgG antibodies to cytomegalovirus (CMV) were measured with an ELISA as described previously(23).

5.3.5 Statistical analyses

We performed the analyses with SPSS version 14 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation, whereas non-parametric variables are given as median (25%-75% interquartile range), and dichotomous variables are in percentages. Serum S100B concentration data were categorized into tertiles for univariate analyses. For linear regression and Cox-regression analyses, the S100B data were log-transformed. We considered a p-value <0.05 for all analyses to indicate significance. Our analyses can be divided into two parts: firstly, we performed cross-sectional analyses to investigate a putative association of CRP and S100B and other potential determinants of serum S100B levels. Secondly, we performed prospective analyses to investigate the association of baseline serum S100B with graft loss.

Potential determinants of S100B were analysed over tertiles of S100B using analysis of variance (ANOVA) for parametric variables, the Jonkheere-Terpstra test for non-parametric variables, and the chi-square-test for dichotomous variables in the cross-sectional part of our analyses. All variables which showed a significant association with S100B were included in further univariable and multivariable linear regression analyses. If baseline variables were highly correlated to each other ($r \geq 0.50$), only the variables with the strongest relation to S100B were included in multivariable linear regression analyses in order to prevent co-linearity and over-adjustment. We excluded variables, which were not significant in the multivariable linear regression model stepwise in order to establish a best-fit model. After the establishment of the best-fit model the excluded variables were reintroduced one by one in order to allow for assessment of the effect of adjustments. We judged the effect of adjustment by comparing the (standardized) regression coefficients and p-values of an association before and after adjustment.

We applied Cox-regression analyses for graft loss with log-transformed S100B as determinant in the prospective part of our analyses. We established one univariable model including S100B alone (crude), and several multivariable models in which the potential confounders were added stepwise. For correction, we used variables which showed significant association with S100B as well as graft loss, as those indicate potential confounding. We also established a multivariable model with urinary protein excretion and patient sex, respectively, since these are known risk factors for graft-failure and mortality(24). Interaction terms were included for S100B and other independent variables. Log-minus-log curves were calculated to investigate the adherence to the proportional hazard assumption.

5.4 Results

5.4.1 Cross-sectional analyses

A total of 581 outpatients (age 52 ± 12 years, 46.0% females, creatinine clearance 61 ± 22 ml/min) participated at a median time of 6.0 [2.8–11.6] years after transplantation in our study. Overall S100B levels were 0.19 [0.14–0.25] µg/L. Baseline characteristics stratified on S100B tertiles are shown in table 1. Among others, patient age, BMI, HbA1c, creatinine clearance, CRP, procalcitonin, sVCAM-1, prevalence of use of statins and prevalence of use of proliferation inhibitors differed significantly between tertiles of S100B. The results of the univariable and multivariable linear regression analyses of significant baseline parameters are shown in table 2. All significant variables from the initial analyses were also at least borderline significant in these analyses ($p \leq 0.2$), except proliferation inhibitor usage. From the significant univariable associations only patient age, BMI, and creatinine clearance appeared to be independently associated with S100B. The linear relation of these variables with log-transformed S100B levels is shown in Figure 1. After step-wise inclusion of variables into the multivariable linear regression model, it appeared that the associations of CRP and HbA1c with S100B became insignificant after inclusion of BMI into the model. Patient sex became insignificant after inclusion of BMI and creatinine clearance. The associations of procalcitonin and sVCAM-1 with S100B became insignificant after inclusion of creatinine clearance.

Table 1: *Baseline characteristics over S100B tertiles*

	s100b tertiles			
	I	II	III	P
n	194	194	194	
Range of s100b conc. (µg/l)	0.00-0.15	0.15-0.22	0.22-2.14	
Demographics				
Age patient (yr)	49 ± 12	53 ± 12	53 ± 12	<0.001
Age donor (yr)	35 ± 15	37 ± 15	39 ± 16	0.2
Sex patient (male; n %)	119 (61.7)	105 (54.1)	90 (46.4)	0.01
Sex donor (male; n %)	106 (54.7)	108 (55.7)	103 (53.4)	0.9
Time since transpl. (yr)	6.1 [3.1-11.8]	7.1 [3.5-12.2]	5.4 [2.3-10.3]	0.1
Smoking, current (n, %)	44 (22.7)	45 (23.2)	40 (20.6)	0.8
Body composition recipient				
BMI (kg/m²)	25.2 ± 4	25.5 ± 4	27.3 ± 4	<0.001
Waist circumf. Women (cm)	90 ± 14	93 ± 14	99 ± 14	<0.001
Waist circumf. Men (cm)	98 ± 14	99 ± 11	103 ± 12	0.01
Hypertension parameters				
SBP (mmHg)	149 ± 22	156 ± 23	154 ± 23	0.05
DBP (mmHg)	88 ± 10	91 ± 10	90 ± 10	0.2
Antihypertens. Med. (n, %)	152 (78.8)	163 (84.0)	159 (82.0)	0.4
ACEi or ATII anta. (n, %)	69 (35.6)	63 (32.5)	62 (32.0)	0.7
Beta-blocker usage (n, %)	108 (56.0)	130 (67.0)	117 (60.3)	0.1
Hyperglycaemia parameters				
Glucose (mmol/l)	4.5 [4.1-5.0]	4.6 [4.1-5.0]	4.6 [4.1-5.1]	0.2
Insulin (µU/ml)	10.8 [7.7-15.2]	11.3 [8.5-15.3]	11.3 [8.0-16.8]	0.7
Diabetes mellitus (n, %)	23 (11.9)	41 (21.1)	39 (20.1)	0.03
HbA1c (%)	6.3 ± 1.0	6.6 ± 1.0	6.6 ± 1.1	0.001
Use of anti-diabetics (n, %)	20 (10.4)	30 (15.5)	28 (14.4)	0.3
History of CVD				
CVD history (n, %)	24 (12.4)	28 (14.4)	20 (10.3)	0.5
Antiplatelet drugs (n, %)	33 (17.1)	41 (21.1)	38 (19.6)	0.6
Lipid parameters				
Total cholesterol (mmol/l)	5.61 [4.98-6.16]	5.45 [4.80-6.14]	5.69 [5.09-6.20]	0.3
HDL cholesterol (mmol/l)	1.06 [0.89-1.30]	1.05 [0.84-1.28]	1.06 [0.87-1.30]	0.8

Table 1 (continued)	I	II	III	P
Triglycerides (mmol/l)	1.90 [1.32-2.64]	1.92 [1.48-2.58]	1.90 [1.41-2.70]	0.5
Use of statines (n, %)	77 (39.9)	102 (52.6)	105 (54.1)	0.01
History of kidney failure				
Previous transplant. (n,%)	6 (5.8)	8 (8.4)	6 (6.6)	0.8
Dialysis duration (months)	24 [12-45]	29 [13-52]	29 [16-51]	0.1
Allograft viability				
Living donor (n, %)	36 (18.7)	22 (11.3)	17 (8.8)	0.01
Warm ischemia (min)	35 [30-43]	36 [30-45]	36 [30-45]	0.05
Cold ischemia (h)	21 [13-26]	22 [15-27]	22 [17-27]	0.2
Acute rejection (n, %)	76 (40.0)	85 (43.8)	76 (39.6)	0.6
Allograft function				
Serum creatinine (µmol/l)	127 [107-148]	140 [110-170]	141 [122-172]	<0.001
Creat. Clearance (ml/min)	66 ± 22	59 ± 21	59 ± 22	0.01
Protein excr. (g/24h)	0.2 [0.0-0.4]	0.3 [0.0-0.6]	0.2 [0.1-0.5]	0.1
Creatinine excretion	12.1 ± 3.6	11.7 ± 3.3	12.0 ± 3.6	0.6
HLA mismatches (n, %)				
HLA AB mismatch	137 (71.0)	142 (73.2)	140 (72.2)	0.9
HLA DR mismatch	68 (36.2)	70 (36.8)	70 (36.8)	1.0
Immunosuppression therapy (n, %)				
Calcineurin inhibitors	144 (74.6)	151 (77.8)	157 (80.9)	0.3
Proliferation inhibitors	152 (78.8)	124 (63.9)	153 (78.9)	0.001
Inflammation				
Albumin (g/l)	41[39-43]	40 [38-42]	41 [39-42]	0.3
hsCRP (mg/l)	1.51 [0.61-3.74]	2.60 [1.01-5.16]	2.43 [1.12-5.99]	<0.001
sRAGE (µg/l)	1.61 [1.20-2.06]	1.72 [1.27-2.34]	1.73 [1.29-2.27]	0.1
Procalcitonin (µg/l)	0.2 [0.2-0.3]	0.2 [0.2-0.4]	0.3 [0.2-0.4]	0.001
sICAM-1 (µg/l)	0.60 [0.51-0.71]	0.61 [0.50-0.73]	0.62 [0.55-0.73]	0.2
sVCAM-1 (µg/l)	0.88 [0.74-1.14]	0.94 [0.76-1.22]	1.02 [0.82-1.24]	0.002
CMV				
CMV patient (n, %)	81 (42.0)	87 (44.8)	101 (52.3)	0.1
CMV donor (n, %)	107 (55.7)	100 (51.5)	105 (54.4)	0.7
CMV IgG, patient (U/ml)	64 [0-141]	70 [0-148]	87 [2-178]	0.02

Figure 1a

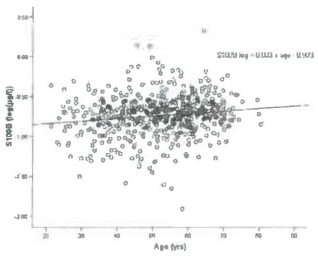

Figure 1b

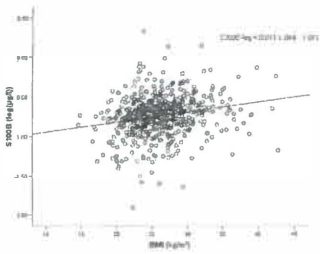

Figure 1c

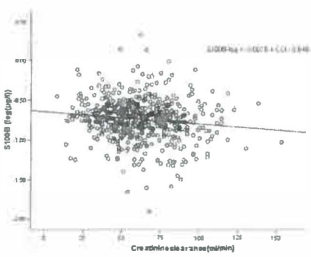

Fig. 1: *Shown are scatter plots of patient age (a), BMI (b) and creatinine clearance (c) in relation to log-transformed S100B levels. The equations represents the best-fit line of the regarding data set. In figure c, CCl is the abbreviation for creatinine clearance.*

5.4.2 Prospective analyses

During follow-up for 5.3 [4.6-5.7] years, n=95 (16.4%) died and n=41 (7.1%) developed graft failure. Levels of S100B were neither significantly associated with graft failure (HR per log(µg/l) = 2.02 [0.62-6.61], p = 0.2), nor with mortality (HR per log(µg/l) = 1.86 [0.85-4.10], p = 0.1). These associations stayed insignificant after correction for the potential confounders found in our analyses and correction for patient sex and urinary protein excretion.

Table 2: Linear regression models for S100B (log(µg/l))

	Univariable			*Multivariable*		
	B	**Beta**	***p***	**B**	**Beta**	***p***
Age patient (yr)	0.003	0.14	0.001	0.002	0.09	0.02
Sex patient (male)	-0.052	-0.10	0.02			
BMI (kg/m²)	0.012	0.20	<0.001	0.013	0.21	<0.001
SBP (mmHg)	0.001	0.10	0.03			
Diabetes mellitus (yes)	0.048	0.07	0.1			
HbA1c (%)	0.024	0.10	0.02			
Statin usage (yes)	0.056	0.10	0.01			
Living donor (yes)	-0.057	-0.07	0.1			
Warm ischemia (min)	0.001	0.06	0.2			
Creatinine clear. (ml/min)	-0.002	-0.13	0.001	-0.002	-0.15	<0.001
Proliferation inhibitor	-0.010	-0.17	0.7			
CRP (log(mg/l))	0.061	0.13	0.002			
Procalcitonin (log((µg/l))	0.098	0.10	0.02			
sVCAM-1 (log(µg/l))	0.184	0.11	0.01			
CMV IgG (U/µl)	0.076	0.06	0.2			

5.5 Discussion

To the best of our knowledge, this is the first study that investigates serum S100B levels in renal transplant recipients. In this study we found serum S100B levels to be independently associated with patient age, BMI and creatinine clearance. No significant association, however, was found with graft failure or mortality.

The relation of serum S100B and age has been reported in healthy individuals(25). The authors of this study describe that serum S100B levels decrease until an age of approx. 35 years and subsequently steadily increase. In the present study we

found a positive linear relationship. Our population matches a subgroup of the above-mentioned study that is aged 35 or above. Thus, the positive relation between age and S100B probably resembles the steady increase of S100B found in the above-mentioned study after the age of 35 years. No study has been published which reports how higher age could lead to higher S100B levels. A possible explanation, however, could be that with increased age there is an increased rate of tissue damage, which results in excretion of S100B.

Reports on a potential association of S100B with BMI are rare. Two studies performed in anorectic patients reported no correlation of S100B with BMI(26;27). However, the BMI values of these study populations were rather in the underweight to normal weight range, whereas the BMI values of our population were in the overweight range, which makes a comparison difficult. Nevertheless, Holtkamp *et al.* did report a correlation between ΔBMI after weight gain and S100B[20]. Some evidence for a potential role of S100B in the fat metabolism comes from animal studies. S100B was found in adipose tissue of rats and it was hypothesized that S100 proteins had a role as transport proteins for fatty acids(28). Another study which investigated the release of S100B from adipocytes suggested that serum S100B levels are significantly influenced by adipose tissue(29).

We found that serum S100B was negatively related to creatinine clearance. A study investigating the elimination of S100B reported that S100B levels were not influenced by a moderate decrease in glomerular filtration rate (GFR)(30). However, the population of this study had rather normal GFR values, whereas our study population had sub-optimal GFR values, which makes a comparison difficult. In general, one might assume that S100B having a molecular weight of 22kDa is filtered by the kidney. Other factors such as extra-renal clearance, however, might also play a role. In another study investigating cell survival after exposure to S100B cytotoxic effects of micromolar S100B levels were found(31). Such a detrimental

effect on kidney tissue could also partly explain the negative association found in our study.

Although the design of this study does not allow causative interpretations, some physiological evidence exists for a potential negative effect of S100B on kidney function. S100B activates an inflammatory response via RAGE(11). RAGE activates the nuclear factor NFκB, which amongst others up-regulates RAGE itself(32). This positive feedback loop may lead to a prolonged inflammatory response resulting in a decline of kidney function and ultimately in graft loss(15).

Some studies have prospectively investigated the association of S100B with mortality in other populations(33;34). In summary, high S100B levels predicted fatal outcome in these studies. However, in our prospective analyses we did not find significant associations of S100B levels with graft failure or mortality.

Our study has some limitations, one of them being the investigated variables, which explain only a small part of the variation of S100B (the R^2 of the multivariable linear regression model = 0.09). It is likely that important factors are missing which could influence S100B levels. Correcting for such determinants could have led to a significant prediction of S100B for graft failure or mortality. Further, the fact that we only measured S100B at one time-point at baseline of our study makes that we can not investigate determinants of changes over time in S100B. Repeated measurements of S100B could have given insight into e.g. whether changes in BMI are associated with changes in S100B. Another limitation is the method we employed to measure S100B levels. This method recognizes the beta-subunit of the S100 proteins, which means that besides S100B proteins S100A proteins were probably also measured. Since S100A proteins activate different pathways than S100B proteins(35), it is possible, that the association of S100B with graft loss was

biased by effects of S100A proteins. Last but not least, our study was designed to represent long-term outcomes rather than acute ones. This could be why we could not find an association between S100B levels and mortality as in the studies on severe brain injury and cardiac failure, in which an acute rise in S100B levels was measured(33;34).

In conclusion, the results of our analyses are in line with a potential (patho)-physiological role of S100B in adipose tissue and the kidney. Our study is the first to show that S100B is associated with BMI in kidney transplant recipients. However, steady-state S100B probably plays no or only a minor role in long-term graft failure and mortality in renal transplant recipients.

5.6 Acknowledgements

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Chapter 6

Influence of C-reactive protein and urinary protein excretion on prediction of graft failure and mortality by serum albumin in renal transplant recipients

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6.1 Abstract

BACKGROUND: Hypoalbuminemia is an established predictor of poor outcome in renal transplant recipients (RTR). It is considered to reflect inflammation, poor nutritional status or proteinuria. We explored the roles of high-sensitivity C-reactive protein (hsCRP) and urinary protein excretion in prediction of graft failure and mortality by serum albumin in RTR.

METHODS: We included 605 RTR at a median [interquartile range] time of 6.0 [2.5-11.5] years after transplantation for baseline measurements.

RESULTS: At baseline, urinary protein excretion (β=-0.242, *P*<0.0001), hsCRP concentration (β=-0.207, *P*<0.0001), recipient age (β=-0.115, *P*=0.004), living kidney donor (β=0.100, *P*=0.01) and a history of myocardial infarction (β=-0.084, *P*=0.03) were independently related to serum albumin. Prospectively, 94 RTR died and 42 suffered graft failure during 5.3 [4.7-5.7] years of follow-up. After adjustment for potential confounders, including hsCRP and urinary protein excretion in Cox-regression analyses, low serum albumin was significantly associated with graft failure [HR=0.34 (95%CI 0.15-0.76) per g/dL, *P*=0.008] and mortality [HR=0.43 (95%CI 0.24-0.78) per g/dL, *P*=0.005], with significant modification of the effect of serum albumin on graft failure by urinary protein excretion (*P*=0.003).

CONCLUSION: Low serum albumin concentrations predict graft failure and mortality in renal transplant recipients independent of hsCRP and urinary protein excretion. The effect of serum albumin on graft failure is strongly modified by urinary protein excretion. These results suggest that chronic low-grade inflammation is not an important mechanism underlying inverse associations of serum albumin with graft failure and mortality. They also suggest that proteinuria is involved in the association of low serum albumin with graft failure.

6.2 Introduction

Hypoalbuminemia is common after renal transplantation (1;2). Serum albumin is a negative acute phase protein, and hypoalbuminemia may therefore be reflecting ongoing chronic low-grade inflammation (3;4). Other potential explanations for hypoalbuminemia are poor nutritional status and loss of protein, in particular with proteinuria (3;4). Low serum albumin has been shown to be a predictor for both graft failure (1;5) and mortality (2;5-7) in renal transplant recipients (RTR). Mechanisms that have been suggested to underlie this association include chronic low-grade inflammation and proteinuria (2;5). Both, proteinuria and inflammation, measured as levels of c-reactive protein (CRP), have also been found to be independent predictors of graft failure and mortality in RTR (8-14). To the best of our knowledge, it is not yet known whether prediction of graft failure and mortality in RTR by serum albumin is independent of high sensitivity CRP (hsCRP) and urinary protein excretion.

In this study we aimed to investigate whether a putative association of low serum albumin with an increased risk for graft failure and mortality is independent of hsCRP and urinary protein excretion which were assessed at the same time.

6.3 Materials & Methods

6.3.1 Study design and patients

In this longitudinal prospective study, all RTR who visited our out-patient clinic between August 2001 and July 2003 and had a functioning graft for at least 1 year were eligible to participate at their next visit to the out-patient clinic. Recipients were asked to participate at a later visit if they were ill or had signs of an infection. A total of 606 RTR signed written informed consent, from an eligible 847 (72%

consent rate). The group that did not sign informed consent was comparable with the group that signed informed consent with respect to age, sex, body mass index, plasma creatinine, creatinine clearance, and proteinuria. Serum albumin concentrations were determined in 605 RTR. Further details of this study have been published previously (13;15-18). The Institutional Review Board approved the study protocol (METc 01/039) which was in adherence to the Declaration of Helsinki. Funding resources had neither a role on the collection and analysis of data, nor in the submission and publication of the manuscript.

6.3.2 Outcome events

All participating subjects visited the out-patient clinic at least once a year. Information on graft failure and mortality was recorded by our renal transplant center and through close collaboration with general practitioners as well as referring nephrologists. Graft failure was defined as the return to dialysis or re-transplantation and was censored for death. Graft failure and mortality of all RTR were recorded until August 2007. There was no loss to follow-up.

6.3.3 Renal transplant characteristics

Relevant transplant characteristics were taken from the Groningen Renal Transplant Database. This database holds information on all renal transplantations performed at our center since 1968, including dialysis history. Standard immunosuppression consisted of the following: prednisolone and Azathioprine (100 mg/day) from 1968 until 1989; cyclosporine standard formulation (Sandimmune, Novartis; 10 mg/kg; trough levels of 175–200 mg/l for the first 3 months, 150 mg/l between 3 and 12 months post-transplant and 100 mg/l thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day) from January 1989 to February 1993. Cyclosporine microemulsion (Neoral; Novartis Pharma b.v., Arnhem, The Netherlands; 10 mg/kg; trough levels idem) and prednisolone

from March 1993 to May 1997. Mycophenolate mofetil (Cellcept; Roche b.v., Woerden, The Netherlands; 2 g/day) was added from May 1997 to date. Current medication and changes in daily prednisolone dose were extracted from the participants' medical records. Delayed graft function was defined as post-transplant oliguria > 1 day.

Body mass index, waist circumference, body surface area (BSA), and blood pressure were measured as described previously(17). Smoking status and cardiovascular history were recorded with a self-report questionnaire. Cardiovascular disease history was considered positive if there was a previous myocardial infarction (MI), transient ischemic attack (TIA) or cerebrovascular accident (CVA).

6.3.4 Baseline laboratory and clinical assessments

Blood was drawn after an 8-12h overnight fasting period. Serum albumin was determined with a Roche Modular P (Roche diagnostics GmbH, Mannheim, Germany) by use of a bromcresol green dye-binding method. Urinary protein excretion was analyzed using the Biuret reaction (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany) and proteinuria was defined as urinary protein excretion ≥0.5 g/24hr. High sensitivity C-reactive protein (hsCRP) was determined using in-house enzyme-linked immunosorbent assays as described before the lowest limit of detection was 0.002 mg/l (19). Total cholesterol was determined using the CHOD PAP method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Low density lipoprotein (LDL) was calculated using the Friedewald formula. High density lipoprotein cholesterol (HDLc) was determined using the CHOD PAP method on a Technikon RA-1000 (Bayer Diagnostics b.v., Mijdrecht, The Netherlands). Plasma glucose was measured by the glucose-oxidase method (YSI 2300 Stat plus; Yellow Springs, OH, USA). Serum creatinine levels were determined using a modified version of the Jaffé method (MEGA AU 510, Merck

Diagnostica, Darmstadt, Germany). Renal allograft function was assessed as creatinine clearance from 24-h urinary creatinine excretion and serum creatinine concentration.

6.3.5 Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) and Sigma Plot version 10 (Systat software Inc., Germany). Parametric variables are given as means ± standard deviation (SD), whereas non-parametric variables are given as median [interquartile range]. Hazard ratios (HR) are reported with 95% confidence interval (CI). A two-sided *P*-value less than $P<0.05$ indicated statistical significance.

First, we analysed recipient or kidney transplant related characteristics according to tertiles of serum albumin concentration in order to investigate which of these factors were associated with albumin concentrations: 1st tertile: 1.2-3.9 g/dL, 2nd tertile: 4.0-4.1 g/dL, and 3rd tertile: 4.2-4.9 g/dL. Differences between the tertiles were tested for statistical significance with one-way analysis of variance for normally distributed variables, Kruskal-Wallis test for skewed distribution, and chi-square test for categorical variables. Subsequently, we determined which variables are independently related to serum albumin concentrations using backward linear regression analysis. All characteristics with a *P*-value ≤0.1 across tertiles of serum albumin concentration were entered into a backward linear regression analysis and removed in successive steps at a threshold of *P*-value ≤0.05. Retained variables were considered to be independently related to serum albumin concentration.

To analyze serum albumin as potential predictor of graft failure and mortality, we first performed Kaplan-Meier analyses with a Log Rank test. Cox proportional hazard regression was used to estimate the effect of serum albumin on graft failure

and mortality. In the multivariate analyses, the associations of serum albumin with both graft failure and mortality were adjusted for recipient age and sex (model 2), for time between transplantation and inclusion date and for creatinine clearance (model 3), for hsCRP (model 4), for urinary protein excretion (model 5), and finally for other variables independently related to serum albumin concentration (model 6), and lastly for risk factors for graft failure and mortality (smoking, mean systolic blood pressure, body mass index, diabetes mellitus, use of calcineurin inhibitor, acute rejection, and delayed graft function) and change in daily prednisolone dose. Secondary analyses were performed with proteinuria as a continuous variable.

Finally, we investigated whether there was an interaction between serum albumin concentration and urinary protein excretion in predicting graft failure and mortality. The interactions were tested by entering serum albumin and urinary protein excretion and their product term in Cox-regression analyses as continuous variables. HRs were also reported according to tertiles of serum albumin and proteinuria (<0.5 g/24hr versus ≥0.5 g/24hr) to allow for interpretation.

6.4 Results

Table 1: *Baseline characteristics according to tertiles of serum albumin concentration.*

	Tertiles of albumin			
	1st	2nd	3rd	*P*
n (%)	218 (36)	155 (26)	232 (38)	
Albumin (g/dL)	3.7 ± 0.3	4.1 ± 0.1	4.4 ± 0.2	
Recipient demographics				
Age (years)	52.9 ± 12.2	53.3 ± 11.5	48.9 ± 12.1	0.0002
Male gender, n (%)	113 (52)	85 (55)	133 (57)	0.5
Body composition measurements				
BMI (kg/m^2)	26.3 ± 4.5	26.4 ± 4.2	25.6 ± 4.1	0.1
Waist circumference (cm)	98.5 ± 14.2	97.8 ± 13.2	95.4 ± 13.5	0.04
Blood pressure				
Systolic pressure (mmHg)	155.9 ± 24.2	152.4 ± 22.3	150.7 ± 21.5	0.04
Diastolic pressure (mmHg)	90.3 ± 10.1	89.3 ± 10.1	90.0 ± 9.6	0.6
Use of ACE-inhibitor or AII-antagonist, n (%)	78 (36)	50 (32)	73 (32)	0.6
Number of antihypertensives	2.0 [1.0+3.0]	2.0 [1.0+3.0]	2.0 [1.0+3.0]	0.3
Prior history of cardiovascular disease				
MI[a], n (%)	23 (11)	15 (10)	9 (4)	0.02
TIA/CVA[b], n (%)	13 (6)	13 (9)	7 (3)	0.07
Diabetes				
Glucose (mmol/L)	4.6 [4.1-5.2]	4.6 [4.2-5.1]	4.5 []4.1-4.9]	0.7
Insuline (µmol/L)	11.4 [7.8-16.3]	10.4 [7.8-16.5]	11.3 [8.5-15.7]	0.6
Diabetes after transplantation, n (%)	50 (23)	27 (17)	30 (13)	0.02
Use of antidiabetic drugs (%)	35 (16)	24 (16)	21 (9)	0.06
hsCRP (mg/L)	3.5 [1.4-8.2]	2.1 [1.0-4.4]	1.4 [0.6-2.9]	0.002
Donor demographics				
Age (years)	36.3 ± 15.5	38.0 ± 15.2	36.8 ± 15.6	0.6
Male gender, n (%)	130 (60)	83 (54)	114 (50)	0.1

Table 1 (continued)	1st	2nd	3rd	*P*
Time between transplantation and baseline (years)	7.2 [4.1-12.3]	6.0 [2.2-11.6]	4.6 [2.4-9.5]	0.07
Renal allograft function				
Serum creatinine concentration (µmol/L)	138 [114-173]	136 [116-169]	129 [106-151]	0.9
Creatinine clearance (mL/min)	58.0 ± 22.7	60.2 ± 21.2	67.0 ± 22.2	<0.0001
Urinary protein excretion (g/24hr	0.7 ± 1.4	0.4 ± 0.5	0.3 ± 0.4	<0.0001
Proteinuria				
<0.5 g/24hr	138 (64)	112 (72)	434 (72)	
0.5-1.0 g/24hr	42 (19)	33 (21)	113 (19)	<0.0001
>1.0 g/24hr	36 (17)	10 (7)	56 (9)	
Prior dialysis duration (mo)	24.0 [11.8-46.3]	30.0 [16.0-50.0]	29.0 [13.0-50.0]	0.06
Transplantation type, n (%)				
Postmortem donor	196 (90)	138 (89)	188 (81)	0.01
Living donor	22 (10)	17 (11)	44 (19)	
Acute rejection, n (%)	94 (43)	76 (49)	101 (44)	0.5
Immunosuppresion				
Prednisolone dose, (mg/day)	10.0 [7.5-10.0]	10.0 [7.5-10.0]	10.0 [7.8-10.0]	0.5
Calcineurine inhibitor, n (%)	175 (80)	120 (77)	179 (77)	0.7
Proliferation inhibitor, n (%)	146 (67)	116 (75)	186 (80)	0.006

Table 1:Values are presented as mean ± standard deviation, median [interquartile range] or percentages. Differences between the tertiles were tested for statistical significance with one-way analysis of variance for normally distributed variables (log-transformation was applied for variables with a skewed distribution), Kruskal-Wallis test for skewed distribution, and chi-square test for categorical variables. Abbreviations: (a) MI: myocardial infarction, (b) TIA: Transient Ischemic Attack, and CVA: Cerebrovascular Accident.

A total of 605 RTR were analyzed at 6.0 [2.6-11.5] years after transplantation. Serum albumin was 4.1 ± 0.3 g/dL. Baseline characteristics according to tertiles of serum albumin are shown in table 1. Cross-sectionally, recipient age, waist circumference, systolic blood pressure, history of myocardial infarction, diabetes, hsCRP and urinary protein excretion were inversely related to serum albumin, whereas positive associations were present for creatinine clearance and use of proliferation inhibitors. There was no association between daily prednisolone dose and serum albumin. The inverse associations of serum albumin concentration with hsCRP concentration and urinary protein excretion are visualized in figure 1. In a multivariate linear regression analysis, recipient age, transplantation with a living donor and a history of myocardial infarction remained independently associated with serum albumin concentration in addition to hsCRP and urinary protein excretion (table 2).

Table 2: *Independent associates and determinants of serum albumin concentration.*

Variable	Standardized Beta	*P*
Urinary protein excretion (g/24hr)	-0.242	<0.0001
hsCRP concentration (mg/L)	-0.207	<0.0001
Age recipient (years)	-0.115	0.004
Transplantation type (postmortem vs living donor)	0.100	0.01
Myocardial infarction (no vs yes)	-0.084	0.03
R^2=0.15		

Variables are listed in order of strength of association according to absolute value of the standardized Beta.

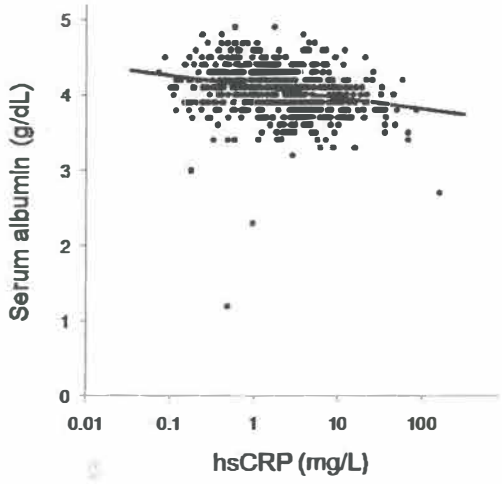

Fig. 1a: *Scatter-plot of serum albumin concentration versus hsCRP concentration. The association best fitted a linear model (serum albumin concentration = 4.10 - 0.15 * Log(hsCRP concentration), P<0.0001.*

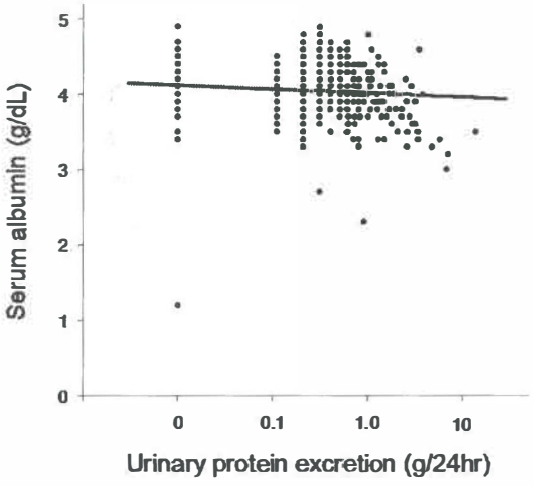

Fig. 1b: *Scatter-plot of serum albumin concentration versus urinary protein excretion. Both associations best fitted a linear model serum albumin concentration= 4.01 - 0.05 * Log(urinary protein excretion), P=0.02).*

Prospectively, 42 (7%) RTR experienced graft failure and 94 (16%) RTR died during follow-up for 5.3 [4.7-5.7] years. Baseline serum albumin at inclusion was significantly lower in RTR who developed graft failure during follow-up than in RTR who did not develop graft failure (3.9 ± 0.3 g/dL versus 4.4 ± 0.3 g/dL, *P*<0.0004).

RTR who died during follow-up also had significantly lower baseline concentrations of serum albumin than RTR who survived during follow-up (3.9 ± 0.3 versus 4.1 ± 0.3 g/dL, *P*<0.0001). During follow-up, daily prednisolone was stopped in 6 (1.0 %) RTR at 1.7 [1.0-3.3] years after inclusion. In 109 (18.0%) other RTR, prednisolone dose was reduced by 25 [17 - 33] % from 10.0 [7.5 - 10] mg/day at 2.0 [0.8-3.9] years after inclusion.

Table 3: *Univariate and multivariate Cox regression analyses for late graft failure and mortality in RTR.*

	Late graft failure		**Mortality**	
	HR [95% CI]	*P*	HR [95% CI]	*P*
Model 1	0.35 [0.22-0.58]	<0.0001	0.39 [0.27-0.56]	<0.0001
Model 2	0.36 [0.23-0.58]	<0.0001	0.36 [0.23-0.56]	<0.0001
Model 3	0.27 [0.14-0.51]	<0.0001	0.39 [0.23-0.66]	<0.0005
Model 4	0.27 [0.14-0.50]	<0.0001	0.41 [0.23-0.71]	0.001
Model 5	0.36 [0.17-0.77]	0.009	0.43 [0.24-0.77]	0.005
Model 6	0.34 [0.15-0.76]	0.008	0.43 [0.24-0.78]	0.005

Serum albumin concentration was entered as the dependent variable in univariate and multivariate analyses.

Model 1: Crude model.

Model 2: Model 1 + adjustments for recipient age and sex.

Model 3: Model 2 + adjustments for creatinine clearance and time between transplantation and baseline.

Model 4: Model 3 + adjustment for hsCRP concentration.

Model 5: model 4 + adjustment for urinary protein excretion.

Model 6: model 5 + adjustment for the other independent associates and determinants of serum albumin concentration.

In the highest tertile of serum albumin, 8 (3%) RTR experienced graft failure during follow-up, whereas these numbers were 9 (6%) and 25 (12%) for the middle and lowest tertile respectively (Log-Rank test: P=0.001, figure 2A). Corresponding numbers for mortality were 19 (8%), 25 (16%), and 50 (23%) (Log-Rank test: P<0.0001, figure 2B). Results of univariate and multivariate Cox regression analyses for graft failure and mortality are shown in table 3. Serum albumin was inversely associated with both graft failure (HR=0.35, P<0.0001) and mortality (HR=0.39, P<0.0001, table 3, model 1). These associations remained significant after adjustment for recipient age and sex (table 3, model 2) and subsequent adjustment for creatinine clearance and for time between transplantation and inclusion date (table 3, model 3). Further adjustment for hsCRP did not affect strength of the association of serum albumin with graft failure, whereas it slightly weakened the association with mortality (table 3, model 4). After adjustment for proteinuria the association of serum albumin with graft failure lost strength, but remained significant (table 3, model 5). Adjustment for proteinuria did not materially change the strength of the association between serum albumin and mortality (table 3, model 5). Final adjustment for the other variables independently related to serum albumin did not materially change the strength of the associations of serum albumin with graft failure and mortality (table 3, model 6). Adjustment for hsCRP and subsequent adjustment for urinary protein excretion did not materially change the association of serum albumin with graft failure and mortality (table 3, model 4 and 5, respectively). The same was true for adjustment for variables independently related to serum albumin (table 3, model 6). Adjustment for smoking, mean systolic blood pressure, body mass index, waist circumference, diabetes mellitus, use of calcineurin inhibitors, acute rejection, delayed graft function and change in daily prednisolone dose during follow-up did also not materially change the association of serum albumin with graft failure and mortality (table 3, model 7). As secondary analyses, we repeated all analyses with proteinuria as a continuous variable. This did not materially affect results of analyses.

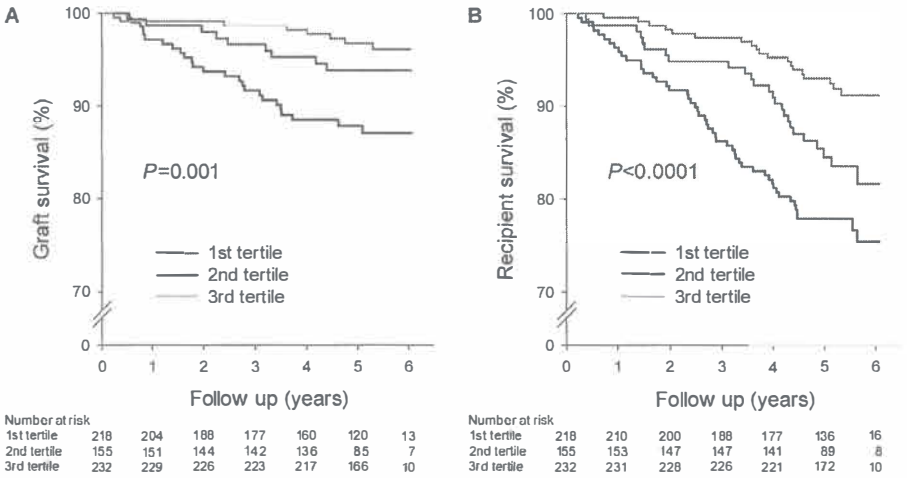

Fig. 2: *Kaplan Meier curves for (A) graft survival and (B) recipient survival in tertiles of serum albumin concentration. Cut-off points for serum albumin concentration: 1st tertile: 1.2-3.9 g/dL, 2nd tertile: 4.0-4.1 g/dL, and 3rd tertile: 4.2-4.9 g/dL.*

We found significant interaction between serum albumin and urinary protein excretion (P=0.003) for prediction of graft failure. There was no significant interaction between serum albumin and urinary protein excretion (P=0.2) for prediction of mortality. Results of Cox regression analyses for prediction of (A) graft failure and (B) mortality by tertiles of serum albumin stratified for urinary protein excretion (<0.5 g/24hr versus ≥0.5 g/24hr) are shown in figure 3. Number of events, hazard ratios and 95% confidence intervals corresponding to figure 3 are shown in table 4. It is clear that the interaction between serum albumin and proteinuria for prediction of graft failure is dictated by proteinuria: without proteinuria there is no association between serum albumin and graft failure. There was only an increase in risk of decreasing serum albumin concentrations in the subgroup with proteinuria. RTR with intermediate concentration of serum albumin and proteinuria (HR=5.7, P<0.005) and RTR with low concentration of serum

albumin and with proteinuria (HR=13.5, *P*<0.0001) were at significantly increased risk for graft failure compared to the reference group. This can not be explained by very different serum albumin concentrations over tertiles of serum in albumin in nonproteinuric versus proteinuric RTR. In non-proteinuric RTR, mean ± SD concentrations of serum albumin over increasing tertiles of serum albumin were 3.8 ± 0.3, 4.1 ± 0.1 and 4.4 ± 0.2 g/dL respectively, while these were 3.7 ± 0.3, 4.1 ± 0.1 and 4.4 ± 0.2 g/dL respectively over increasing tertiles of serum albumin for proteinuric RTR. It is evident that there was no interaction between serum albumin and urinary protein excretion for prediction of mortality. RTR with low concentrations of serum albumin without proteinuria (HR=2.7, *P*<0.005), RTR with intermediate concentrations of serum albumin with proteinuria (HR=2.8, *P*<0.05), and RTR with low concentrations of serum albumin with proteinuria (HR=3.9, *P*<0.0001) were at increased risk for mortality compared to the reference group.

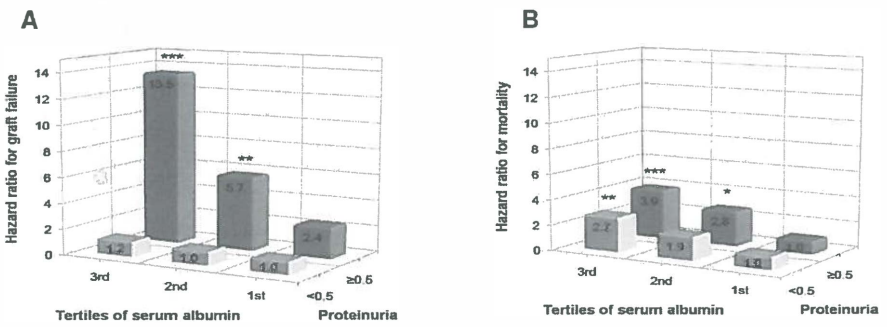

Fig. 3: *Interactions between tertiles of serum albumin concentrations and proteinuria (<0.5 g/24 hr and ≥0.5 g/24 hr) on the risk of (A) graft failure and (B) mortality on the risk of mortality. RTR with high serum albumin concentration and without proteinuria were regarded as reference group. *P less than 0.05, **P less than 0.005, and ***P less than 0.0001 compared to RTR with high serum albumin concentration and low urinary protein concentration.*

6.5 Discussion

In the cross-sectional part of this study, we found independent inverse associations of serum albumin with hsCRP and urinary protein excretion. Prospectively, we found that RTR with low serum albumin concentrations at baseline are at increased risk for graft failure and mortality during follow-up. Despite the cross-sectional associations of serum albumin with hsCRP and urinary protein excretion at baseline, the association of serum albumin with graft failure was not explained by hsCRP, while the association of serum albumin with mortality was explained by hsCRP only to a very small extent (change in hazard ratio from 0.39 to 0.41). Importantly, we found that the association between serum albumin and graft failure was only present in RTR with proteinuria, indicating that low serum albumin is not itself a risk factor for graft failure in RTR. It is more probable that low serum albumin reflects severity of proteinuria in these patients. For mortality, the impact of serum albumin was similar in RTR with and without proteinuria.

We found urinary protein excretion, hsCRP concentration, recipient age, transplantation with a living donor and a history of myocardial infarction to have independent inverse associations with serum albumin concentration. The independent association of recipient age and urinary protein excretion with serum albumin in RTR has been shown before(2). The same is true for the cross-sectional association between serum albumin and a history of myocardial infarction (20;21). To the best of our knowledge, our study is the first to investigate the potential existence of an inverse association between serum albumin and hsCRP in RTR. The inverse association that we found extends observations of such a relationship in other studies, including patients with end-stage renal disease (22;23). Albumin is a negative acute-phase protein and its synthesis is suppressed during inflammation, regardless of nutritional status (24), which could be the

explanation for the observed association between serum albumin and hsCRP. Apart from chronic low-grade inflammation and loss of urinary protein excretion, malnutrition may, however, also play an important role in low albumin concentrations in RTR. We showed that the associations of low serum albumin with graft failure and mortality are independent of BMI, waist circumference, diabetes mellitus, prednisolone dose and changes in daily prednisolone dose during follow-up. Albeit adjustment for these variables is likely to be imperfect for assessment of the influence of nutritional status, it is quite conceivable that nutritional status plays a role in our finding that transplantation with a kidney from a living donor is independently, positively associated with serum albumin.

Our finding of an association of serum albumin with development of graft failure during follow-up after renal transplantation is consistent with existing literature (1;5). Massy *et al.*(1), found that serum albumin (HR equivalent to 0.20 for each g/dL, $P<0.0005$) was an independent predictor of graft failure. This finding was confirmed by Moore *et al.* (5) who also found that serum albumin was an independent predictor of graft failure (HR in multivariate analysis equivalent to 0.54 for each g/L, $P<0.001$). In these studies, potential interaction between serum albumin and urinary protein excretion was not investigated. We found significant interaction between serum albumin concentration and urinary protein excretion in predicting graft failure. The interaction between serum albumin and proteinuria is consistent with a relatively weak or even absent association between serum albumin and graft failure in the absence of proteinuria. This observation suggests that low albumin concentrations are not a strong risk factor for graft failure by themselves. Rather, it suggests that proteinuria plays an important role. One reason may be that severity of proteinuria in some RTR is underestimated as a consequence of errors in collecting 24h urine samples. It is well-known that 24h urine collection is prone to collection errors (25-28). Another reason may be that low albumin concentrations in the presence of proteinuria are an indication of

detrimental effects or urinary loss of peptides undetected by urinary protein assays as a consequence of tubular processing (29-31).

Our finding of an inverse association between serum albumin and mortality in RTR is consistent with existing literature (2). Guijarro *et al* were the first to show that low serum albumin was an independent risk factor for mortality (RR=0.26 for each g/dL). This finding was subsequently corroborated by several other studies (5-7). To the best of our knowledge, our study is the first to investigate whether serum albumin predicts mortality in RTR independent of hsCRP. Despite the baseline association between serum albumin and hsCRP, the association of serum albumin with mortality was not materially affected by adjustment for hsCRP. Many studies have suggested that chronic low-grade inflammation is likely to be one of the mechanisms underlying the association of low serum albumin with increased risk for mortality (2;5). Our finding suggests that either chronic low-grade inflammation should be measured by other markers or that other mechanisms are involved. It would also be of additional value to use repeated measurements of hsCRP. However, since we only assessed hsCRP concentrations at baseline this was not possible. Our multivariate analyses show that, besides of hsCRP, several other factors, including age, sex, creatinine clearance, history of cardiovascular disease, smoking and diabetes mellitus are unlikely to be important confounders of the association of serum albumin with mortality. Effects of proteinuria are also unlikely to be involved in this association, because it is also independent of urinary protein excretion. Another possibility would be malnutrition. It has been reported that malnutrition occurs in 15-20% of the RTR (4;32). Malnutrition as partly reflected by low albumin concentrations is a powerful risk factor for morbidity and mortality in dialysis patients (33-35). Since virtually all RTR are treated with corticosteroids, decreased synthesis and increased catabolism of albumin secondary to the use of corticosteroids could also play a role (36). However, we did not find an association between daily prednisolone dose and serum albumin. Furthermore, adjustment for

change in daily prednisolone dose during follow-up did not materially change the association of low serum albumin with increased risk for graft failure or mortality.

A possible limitation of our study is that we did not use repeated measurements of albumin concentrations in our analyses. However, most epidemiological studies, such as previous studies on albumin concentrations as predictor of events in RTR (1,2,5-7), use single baseline measurements to predict outcomes. Use of a single value instead of repeated measurements adversely affects predictive properties, because of taking into account intra-individual variability of predictive parameters results in stronger relations with outcomes (37;38). Therefore, associations for albumin with outcome would probably have even been stronger if we could have used repeated measurements in our analyses. However, this was not possible, because measurement of variables, including hsCRP for which we aimed to adjust, was only assessed at baseline and not repeatedly. An important strength of our study is that there was no loss to follow-up. Besides the studied variables, which may confound the effect of hypoalbuminemia, other potential confounders exist. These include the effect of overhydration influencing albumin levels; the effect of hypoalbuminemia on drug binding, exposure and metabolism, the effect of hypoalbuminemia interfering with the measured creatinine levels in Jaffé-based creatinine assays which could artificially change the creatinine clearance estimations(39). However, we did not measure these variables. This might be an interesting subject for future research. Another limitation worth mentioning is the prevalent nature of the population, leading to "survivor bias" being a potential confounder. We tried to adjust for this by adjusting for time post transplantation in multivariate analysis. However, this does not fully abrogate the “survivor bias” as a potential confounder.

In conclusion, low serum albumin late after renal transplantation is a predictor of graft failure and mortality, independent of hsCRP and urinary protein excretion. The effect of serum albumin on graft failure was strongly modified by urinary

protein excretion. Although an association between low serum albumin and decline of renal function can not be excluded, low serum albumin may reflect damaging effects of proteinuria on the transplanted kidney. Because our results suggest that inflammation is not responsible for the association between serum albumin and mortality, we speculate that malnutrition might be a pathway linking serum albumin to mortality. Future studies are needed to investigate whether nutritional and non-nutritional interventions can improve serum albumin concentration among RTR and whether an improvement in serum albumin can lead to reduced mortality.

6.6 Acknowledgements

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Chapter 7

Discussion and future perspectives

Sascha Gross

In this thesis it was investigated whether advanced glycation-related markers could predict long-term outcome after renal transplantation, i.e. graft failure and mortality. The focus was on advanced glycation end-products (AGEs) and their receptor (RAGE) and a potential role of inflammation. In this thesis it was found that low serum levels of soluble RAGE (sRAGE), a potential AGE neutralizer, were associated with higher risk for mortality. Further, it was found that skin autofluorescence (skin AF), a marker for endogenous AGE-accumulation, could predict outcome and that serum levels of CML, the most prominent AGE in humans, could predict mortality. No significant relation was found between steady-state S100B, an endogenous ligand of RAGE, and outcome. Last but not least, low albumin levels, a potential indicator for chronic low-grade inflammation, showed significant associations with graft failure and mortality. In supplementary analyses it has been found that the associations of sRAGE, skin AF and CML were largely independent of inflammatory markers including hsCRP (data not shown).

7.1 Low levels of sRAGE are associated with mortality

In **chapter 2** which investigated the association of serum sRAGE with outcome, low levels of sRAGE were associated with higher risk for mortality. Since sRAGE lacks the signal domain of RAGE, it has been hypothesized that sRAGE neutralizes AGEs, prevents RAGE signalling and thus has a protective effect in terms of AGE-related pathologies. In murine models application of sRAGE, indeed, had beneficial effects for the development of atherosclerosis(1). However, high sRAGE levels also correlated with low kidney function in these analyses, which would rather indicate that sRAGE has detrimental effects on kidney function. An alternative explanation for this inverse relation could be that sRAGE is cleared by the kidney and that it accumulates with low kidney function. This idea was suggested by Kalusova *et al.*, who also found an inverse relation between sRAGE and creatinine clearance in patients with low kidney function(2). In our study we did

not find a significant association of sRAGE with graft failure. Although the model was statistically not significant, correction for creatinine clearance showed a strong effect on the hazard ratios of sRAGE which could point to a potential association of sRAGE with a lower risk of graft failure. A study examining serum AGE and sRAGE levels in older community-dwelling women found significant associations of both AGEs and sRAGE with kidney function(3). This further supports the idea that sRAGE could actually be associated with graft failure in renal transplant recipients and that not finding this association in renal transplant recipients could be due to a low effect size of sRAGE in this study. A function of sRAGE as a decoy receptor, however, is questionable. Besides its potentially protective role sRAGE might also be a bystander product and reflect RAGE activity. This could be a reason why we found low sRAGE to be a risk factor, but not high sRAGE to be protective. In the setting of uremia it seems more likely that sRAGE reflects RAGE activity, since in uremia sRAGE might not be cleared well by the kidney. Further, it was recently found that sRAGE might also have pro-inflammatory characteristics by interaction with the integrin Mac-1(4). In the end it is also not clear whether the sRAGE proteins measured by ELISA are free sRAGE molecules, which are still able to bind AGEs or whether these are sRAGE molecules which have bound AGEs, or both. In order to bring more light into the sRAGE mystery, future research would have to address these issues.

7.2 AGEs are associated with mortality

Chapter 3 in which skin AF was measured, showed that AGEs found in skin predict graft loss including mortality and **chapter 4** in which serum CML was measured showed that soluble AGEs can predict mortality after renal transplantation. These results are in line with the current paradigm that AGEs have detrimental effects and may cause cardiovascular disease(5). AGEs can act in receptor-independent ways by e.g. cross-linking endogenous proteins and by

receptor activation(6;7). RAGE is the most prominent receptor of AGEs and an activation of RAGE has been hypothesized to lead to sustained inflammation(8). The associations of skin AF and CML with mortality were found to be independent of inflammation markers. This may indicate that receptor-independent pathways play a more important role than receptor-dependent, inflammatory pathways.

Several studies showed associations of AGEs and RAGE with chronic disorders and the clinical relevance of AGEs is growing(5). Especially in heart and kidney disease AGEs are more and more considered to play an important role(9). While there are several tests available for the measurement of AGEs and currently AGE-lowering drugs are investigated, no clinical trials involving AGE-related risk are currently performed after renal transplantation. The studies investigating skin AF and plasma CML show the importance of including AGEs in the risk estimation of renal transplantation. Potential future procedures including AGE measurements and subsequent AGE removal may benefit long-term outcome of renal transplantations.

7.3 S100B is not significantly associated with graft failure or mortality

Chapter 5 in which S100B was measured did not show a significant association of S100B and outcome in the same study population. S100B is a ligand of RAGE which may have similar importance in neuronal disorders as CRP in inflammation(10). Recently, evidence has grown for S100B to also play a role in renal tissue(11). An association of S100B with outcome could have pointed to a potential role of RAGE activation by S100B in the pathophysiology of graft failure and mortality. The absence of this association, however, may have many explanations besides a potential low importance of the RAGE axis in long-term

outcome of renal transplantation. For example S100B is known to have an ambivalent character. In micromolar concentrations S100B has toxic effects, while in nanomolar concentrations it has trophic effects(12). In our study serum S100B levels were in the nanomolar range. However, these levels could relate to higher levels in the kidney. It could therefore be that S100B in our study reflects both trophic and toxic effects which in the end cancel each other out.

In studies investigating the predictive value of S100B levels for mortality, S100B levels were measured shortly after acute events, while in our study steady state levels of S100B were measured. In the studies, which measured acute levels of S100B, S100B did predict mortality(13;14). Although the different study populations may play a role, also the difference in acute and steady state S100B levels could be of importance. Acute S100B levels are high in general and evidence exists that S100B represents tissue damage under these conditions. This is supported by the finding that S100B is released with metabolic stress and after tissue injury(10;15). Steady state S100B levels, however, might be lower and rather represent a more physiological character of S100B. In neuronal tissue it has been shown that S100B regulates cell survival(16). This ambivalent character of S100B might be responsible for not finding a significant association of S100B with graft failure or mortality. On the other hand, a physiological role of S100B has not been shown in tissues other than neuronal tissue, which would not contradict the initial hypothesis, while it has been shown that S100B activates pro-inflammatory RAGE signalling(17;18). Therefore, not finding a significant association between steady-state S100B levels and outcome could also indicate that the non-AGE-related activation of RAGE might play a minor role compared to AGE-related activation

7.4 Serum albumin is associated with graft failure and mortality

Chapter 6 in which serum albumin was measured showed that serum albumin predicted graft failure if proteinuria was present. Further, low albumin predicted mortality independent of proteinuria. Both the association of serum albumin with graft failure and mortality were independent of hsCRP.

Proteinuria, inflammation and nutrition determine serum albumin levels(19). Further, serum albumin is associated with graft loss and mortality in renal transplant recipients(20;21). In this study we found that the association of serum albumin with graft failure depended on proteinuria. This means that low serum albumin levels probably reflect proteinuria, which is a risk factor for graft loss. Since proteinuria is not as strongly related to mortality, it probably does not confound the association of albumin with mortality.

The association of albumin with mortality was independent of hsCRP. This may suggest that albumin represents inflammation better than hsCRP, or that albumin has a relation to mortality which does not involve inflammation. A possible link might be nutrition. Low albumin levels reflect low nutrition, which could be a risk factor for mortality. Another possibility might be the function of toxin removal by albumin. Low albumin levels would then reflect a low potential of toxin removal, which could also be a risk factor for mortality.

However, an association independent from hsCRP may also indicate that inflammation might not sufficiently be represented by serum albumin levels or that markers other than hsCRP might have better represented the type of inflammation involved. In several studies an inverse relation between serum albumin and

inflammation was found(19). Further, in hemodialysis patients it was found that the association between serum albumin and mortality was partly explained by inflammation but not by malnutrition(22). This supports the idea that inflammation may be sufficiently represented by low serum albumin levels in kidney transplant recipients. Further, in a study investigating hsCRP, IL-6 and serum albumin as prospective markers for CVD in patients with end-stage renal disease it was found that hsCRP did not independently predict CVD while IL-6 did(23). Together this may indicate that inflammation markers other than hsCRP could have explained part of the association between serum albumin and mortality in our study.

7.5 The role of AGE-related inflammation

In supplementary analyses (data not shown) several inflammatory markers were measured and their relation to the AGE-related markers (sRAGE, skin AF, CML, S100B) was investigated. From these analyses it turned out that each of the markers were related to inflammatory markers. However they differed in their specific relation. E.g. skin AF was independently associated with VEGF, sE-selectin, sICAM-1 and hsCRP, while CML was independently associated with number of leucocytes, s-VCAM-1, procalcitonin and hsCRP.

Although all AGE markers were related to inflammation their prospective association with outcome was independent of inflammation. This may suggest that inflammation may not play a crucial role in advanced glycation-related pathologies in renal transplantation. It rather seems that direct effects of AGEs may underlie the associations found in these analyses. Supportive to a minor role of inflammation also are the low R^2-values found in these additional analyses. The R^2-values values ranged from 0.05 (sRAGE) to 0.1 (skin AF). This means that

inflammatory markers explain only a small part of the variation in these variables, which makes it likely that there are other, yet unknown, factors which may be more important determinants.

7.6 Future perspectives

In summary, this thesis shows the importance of AGEs in renal transplantation and suggests a future inclusion of AGE-related measurement in the risk assessment for late complications after renal transplantation. The contribution of inflammation in AGE-related pathologies may play a minor role. However, it has been found that inflammation *per se* is predictive for outcome and thus may play an important role independent of advanced glycation.

Given the important role of AGEs in patients with end-stage renal disease this thesis supports further investigation of AGE-removing therapies. Promising results for AGE-breaking drugs have been obtained from animal experiments(24-26). To date safety of these drugs is investigated in patients(27;28). In the near future AGE-breaking drugs might therefore bring improvement in survival of renal transplant recipients. Any drug, however, has unwanted side-effects. Therefore, other strategies of AGE-removal should also be taken into account. For example the AGE-binding potential of sRAGE could be exploited to efficiently remove circulating AGEs from the blood stream. AGEs are not removed efficiently by present renal replacement therapies(29;30). This might be the result of non-specific removal of AGEs. Immobilized sRAGE molecules could be used in a biotechnological approach to enhance the efficiency of currently available dialysis machines.

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Samenvatting

De behoefte aan niertransplantatie zal in de toekomst werelwijd zeer waarschijnlijk toenemen, omdat – mede door veroudering, obesitas en diabetes - meer en meer mensen een chronische nierziekte hebben. Ondanks verbeterde preventieve maatregelen, ontwikkelt een gedeelte van de patienten met een chronische nierziekte nog altijd nierfalen en moet daarvoor worden behandeld door middel van dialyse of niertransplantatie. Niertransplantatie heeft de voorkeur, omdat deze therapie betere uitkomsten geeft qua mortaliteit en levenskwaliteit. Het belangrijkste probleem van niertransplantatie, het binnen een jaar na transplantatie afstoten van het transplantaat, is grotendeels opgelost door verbeterde immunosuppressieve behandeling van patienten.

De lange termijn uitkomsten van nietransplantatie zijn minder tevredenstellend. Patienten met een niertransplantaat hebben een vier- tot zesvoudig verhoogde mortaliteit vergeleken met gezonde mensen en bij ongeveer 50% van de niertransplantatiepatienten ontstaat binnen 10 jaar na transplantatie opnieuw nierfalen.

Advanced glycation end-producten (AGEs) zijn stoffen die niet alleen voorkomen in maaltijden, maar die ook spontaan in het lichaam kunnen ontstaan onder invloed van bijvoorbeeld verhoogde waarden van bloedglucose. In patienten met lage nierfunctie kunnen AGEs verder opstapelen doordat ze minder worden uitgescheiden. Van AGEs wordt gedacht dat ze veel kwalijke eigenschappen hebben, deels door indirecte effecten zoals stimulering van de receptor voor AGEs (RAGE) en deels door directe effecten zoals het onderling verbinden en tot klitten maken van eiwitten en eiwitfragmenten. Hierdoor zou onder andere chronische laaggradige ontsteking kunnen ontstaan wat kan bijdragen aan beschadiging van nieren en bloedvaten. Hierdoor kan uiteindelijk opnieuw nierfalen ontstaan en het kan sterfte aan hart- en vaatziekten veroorzaken.

In dit proefschrif is onderzocht of het in de bloedsomloop aanwezige oplosbare gedeelte van de receptor voor AGEs (sRAGE), S100B (een stofje dat de receptor

voor AGEs kan stimuleren), het serum carboxymethyllysine (CML) (de meest voorkomende AGE), huid fluorescentie (een marker voor stapeling van AGEs in weefsels) en serum albumine (een marker voor inflammatie) mortaliteit of nierfalen kunnen voorspellen.

Alle genoemde markers voorspelden mortaliteit behalve S100B. Dit suggereert dat AGEs een belangrijke risicofaktor zijn voor mortaliteit. Het feit dat S100B niet voorspellend is, suggereert dat stimulering van RAGE hierbij niet zo erg belangrijk is. Dit zou betekenen dat AGEs veeleer werken door directe effecten, zoals cross-linking. Toekomstig onderzoek zal moeten uitwijzen of AGE-breaking medicamenten de mortaliteit van niertransplantatiepatienten kan minderen.

Van de genoemde markers voorspelden huidfluorescentie en serum albumine nierfalen. Echter, serum CML, sRAGE en S100B voorspelden nierfalen niet. De voorspellende waarde van huidfluorescentie en serum albumine was onafhankelijk van inflammatie. Daarom zijn er in dit proefschrift geen sterke aanwijzigingen gevonden voor een toxisch effect van AGEs op de nier, behoudens het feit dat de relatie tussen huidfluorescentie en het ontstaan van nierfalen daarbij zou kunnen passen.

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Sascha

List of Publications

Sascha Gross, Rutger M. van Ree, Leendert H. Oterdoom *et al.* Low Levels of sRAGE Are Associated with Increased Risk for Mortality in Renal Transplant Recipients (*Transplantation.* 2007 Sep 15;84(5):659-63)

Sascha Gross, Jasper W. L. Hartog, Leendert H. Oterdoom *et al.* Skin autofluorescence is an independent predictor of graft loss in renal transplant recipients (*Transplantation.* 2009 Apr 15;87(7):1069-1077)

Sascha Gross, Rutger M. van Ree, Leendert H. Oterdoom *et al.* Body mass index and creatinine clearance are associated with steady-state serum concentrations of the cell damage marker S100B in renal transplant recipients. (*Med Sci Monit.* 2010;16(7):CR318-24.)

Sascha Gross, Jasper W. L. Hartog, Leendert H. Oterdoom *et al.* The advanced glycation endproduct Nε-carboxymethyllysine (CML) is a risk factor for graft failure and mortality in kidney transplant recipients (*submitted*)

Friso L.H. Muntinghe, Sascha Gross, Stephan J.L. Bakker *et al.* CCR5 Δ32 genotype is associated with outcome in type 2 diabetes mellitus (*Diabetes Res Clin Pract.* 2009 Nov;86(2):140-5. Epub 2009 Sep 9)

Rutger M. van Ree, Sascha Gross, Jaap J. Homan van der Heide *et al.* Influence of C-Reactive Protein and Urinary Protein Excretion on Prediction of Graft Failure and Mortality by Serum Albumin in Renal Transplant Recipients (*Transplantation* 2010 May 27;89(10):1247-54)

Henk A. Martens, Sascha Gross, Gerrit van der Steege *et al.* Association of C-C chemokine receptor 5 (CCR5) Δ32 deletion status with rheumatoid arthritis, systemic lupus erythematosus, lupus nephritis and disease severity (*J Rheumatol.* 2010 Aug 3. [Epub ahead of print])

Douwe J. Mulder, Paul L. Van Haelst, Sascha Gross *et al.* Skin autofluorescence is elevated in patients with stable coronary artery disease and is associated with serum levels of neopterin and the soluble receptor for advanced glycation end products (*Atherosclerosis*. 2008 Mar;197(1):217-223)