Novel diagnostics and therapeutics to prevent injury in native and transplanted kidneys

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Table of Contents

Chapter 1

General introduction and outline

The kidneys and development of injury

In physiological conditions, kidneys excrete waste products from the circulation, regulate blood pressure, balance of body fluids, and electrolytes, activate vitamin D for adequate bone mineralization and produce erythropoietin to stimulate red blood cell formation. In case of progressive kidney injury, these functions can become compromised.

Kidney injury is divided into acute and chronic injury. Acute kidney injury is caused by a diversity of conditions, including pre-renal (insufficient perfusion of the kidney), post-renal and renal causes. Chronic kidney disease is defined by the presence of decreased kidney function or kidney damage for at least three months.[1] The assessment of patients with newly diagnosed chronic kidney disease consists of a calculated estimation of the glomerular filtration rate (eGFR), examination of the urine (qualitive tests and microscopic), and, if necessary, serologic testing, radiologic imaging of the kidneys, and kidney biopsy examination. In the majority of cases, chronic kidney injury is irreversible and histological changes are characterized by presence of fibrosis in the kidney.[2] In the United States, 6.7% of the population has a diminished eGFR (<60 ml/min/1.73m2).[3] The most common causes of chronic kidney disease are, amongst others, poorly controlled diabetes mellitus and hypertension. Diabetes mellitus accounts for 30 to 50 percent of patients with end stage renal disease.[4] Consequently, the main aim of treatment for diabetes in an earlier stage is restraining the progression of such complications, by improved glycemic and blood pressure regulation, cardiovascular risk reduction and inhibition of the renin-angiotensin system.

Kidney transplantation and simultaneous pancreas kidney transplantation

In case end-stage renal disease develops, different treatment options are possible; dialysis and kidney transplantation as two renal replacement therapies and conservative treatment consisting of drug and dietary therapy, primarily aiming for optimization of the quality during the final stage of life. The choice for renal replacement therapy depends upon the overall prognosis and condition of the patient. Kidney transplantation is the preferred renal replacement therapy, since kidney transplantation results in better patient survival and improved quality of life.[5,6] Indeed, patient survival is significantly higher in renal recipients, compared to patients on the waiting list that are on dialysis treatment. However, a shortage exists of deceased kidney donors after circulatory death (DCD) or brain death (DBD) and prolongs time on the waiting list for a renal allograft. The number of transplanted kidney grafts from living donors has increased and countervails the shortage of deceased donation. In The Netherlands, the proportion of living kidney donation increased in the last decades to 50% of the 900-1,000 annual kidney transplants.[7] The advantage of living-donor kidney donation is the planning reliability of the transplantation trajectory and a superior graft function and graft survival.[5,8]

In patients with end-stage renal disease due to diabetic nephropathy, simultaneous pancreas-kidney transplantation (SPKT) is the preferred treatment, since SPKT offers

superior long-term survival in patients with diabetes type 1, compared with kidney transplantation alone.[9] SPKT has a deceased donor, since pancreas donation is not possible during life. Next to replacement of kidney function, SPKT restores endogenous insulin secretion and decreases microvascular complications.[10] On a yearly basis, 20-30 SPKTs are performed in the Netherlands.[7]

Although transplantation is the preferred option in many patients with end-stage renal disease, transplantation has disadvantages as well, including a decreased patient survival in the first year after transplantation.[11] Additionally, several factors may limit graft survival. The most prominent causes of graft loss are studied in this thesis, namely (1) rejection and (2) fibrosis and atrophy. In the last decades, the overall graft survival has increased in both the short and long term,[12] although the improvement is mostly caused by better short-term results.[13]

- Rejection is divided into hyperacute, acute and chronic rejection.[14] Hyperacute rejection is due to preformed donor-specific antibodies at the time of transplantation.[15] Frequently, primary non-function is observed and graft loss occurs within 24 hours after transplantation. Hyperacute rejection is rare nowadays, because of improved screening for antibodies. Acute rejection is divided into T cell-mediated rejection and antibody-mediated rejection (ABMR). T cell-mediated rejection is the most common form of acute rejection and is characterized by interstitial inflammation, tubulitis, and sometimes arteritis[16] and is often treated with corticosteroids and/or ATG initially. ABMR is characterized by microvascular inflammation, evidence of antibody interaction with the vascular endothelium and serologic evidence of circulating donor-specific antibodies (DSAs).[16] Although ABMR encompasses only a small proportion of the total number of rejections, the severe decline in kidney function makes ABMR a condition to take into account in the screening and follow up of kidney recipients.[17-19] The presence of preformed DSAs (i.e. DSAs present before transplantation) are associated with the development of ABMR[20] after transplantation and a lower overall graft survival.[21] On the other hand, the evidence for treatment for ABMR is scarce. Plasma exchange, intravenous immune globulin, and glucocorticoids are possible prescribed in patients with ABMR. However, the choice of treatment is based on small studies and expert consensus.[22]

- Chronic damage in a transplanted kidney graft is characterized by interstitial fibrosis and tubular atrophy (IFTA). The process is poorly understood and is typically accompanied by slowly rising serum creatinine concentration and increasing proteinuria. Immunologic factors, such as inflammatory cytokines and cell-mediated and humoral immune responses, are considered to play an important role.[23] An episode of acute rejection would be prognostic for IFTA,[24] although there is no consensus on this subject. Another important factor is the immunosuppressive regimen, since calcineurin inhibitors in

particular, are associated with chronic kidney injury after transplantation.[25] Other factors are glomerular hyperfiltration, delayed graft function, and hyperlipidemia.[26-28]

Other causes, not studied in this thesis, are recurrence of the primary kidney disease (frequently seen in patients with focal segmental glomerulosclerosis, IgA nephropathy, and membranoproliferative glomerulonephritis[29]) and peri-operative complications in the first months after transplantation (i.e. vascular thrombosis, fluid collections, and impaired wound healing[30]).[31,32]

Determination and prevention of kidney injury in native and transplanted kidneys in an early stage

The progression to end-stage renal disease in patients with chronic kidney injury and progression of kidney injury in renal recipients is difficult to predict. In diabetes patients for example, 30 to 40 percent of patients develop diabetic nephropathy, while others show a milder course of deterioration of the kidney function.[4] In transplantation, events that cause kidney injury, such as rejection, occur in a small proportion of recipients and some patients develop more IFTA than others. Identification of these patients is of great importance to personalize prevention and treatment of disease progression. In current monitoring strategies, creatinine clearance and proteinuria play an important role. These, however, are late signs of kidney injury and do not predict further progression of kidney injury. New biomarkers are required to increase understanding of the pathogenesis of kidney injury and thereby recognize progressive injury in an early stage. In addition, different treatment regimens may prevent the progression of injury. In this thesis, we aimed to identify novel markers of vascular and tubular injury in patients with chronic kidney injury and in transplant recipients and improve current diagnostic and therapeutic approaches to prevent kidney injury.

Markers of vascular injury

Regardless of the etiology, both acute and chronic kidney injury involve cellular changes that disturb the delicate renal vasculature.[33] In particular, diabetes mellitus is associated with microvascular injury. In transplantation, microvascular endothelial injury is one of the main features of both acute rejection and chronic injury, previously known as chronic allograft nephropathy.[33] Microvascular injury in the context of kidney disease and cardiovascular diseases have been previously linked to altered levels of specific long noncoding RNAs.[34,35] It is recognized that noncoding RNAs play an important role in molecular mechanisms, such as transcription, splicing and translation.[36] In human, only 1-2% of the genome codes for proteins. The remaining part of the genome does not code for proteins and RNA transcribed from this part is therefore called noncoding RNA[37] while being considered as 'junk' in the past. Several types of noncoding RNA are described, among which microRNA, circular RNA and long noncoding RNA (lncRNA). The latter is the largest group of noncoding RNAs and is characterized by a length of more than 200 nucleotides.

LncRNAs are increasingly described in the context of both glomerular and tubulointerstitial kidney diseases.[38] Next to kidney diseases, lncRNAs are suggested to play an active role in several other vascular diseases.[34] As such, lncRNAs may provide interesting candidates for the detection of early vascular injury in the context of kidney diseases and the vascular status of transplanted renal recipients.

Markers of tubular injury

Next to vascular damage, tubular injury is one of the hallmarks of kidney injury. In order to restrain the negative effects of kidney injury, senescence of cells is induced by the initiation of cell cycle arrest, in particular in tubular cells.[39]

Two rate-limiting factors that regulate the process of cell division and induction of apoptosis are p53 and p27. P53 regulates apoptosis and DNA repair and p27 inhibits cyclins that are necessary for progression through the cell cycle by activation of cyclin-dependent kinase enzymes.[40,41] Interestingly, two novel proteins have been identified that affect p53 and p27. Insulin-like growth factor-binding protein 7 (IGFBP7) is assumed to increase the expression of p53, while IGFBP7 and Tissue inhibitor of metalloproteinase 2 (TIMP-2) increase de novo synthesis and binding capacity of p27, a cyclin-dependent kinase inhibitor.[40,42,43] During episodes of kidney cell injury, G1 cell cycle arrest can be initiated, in order to avoid increased damage due to cell division. Both IGFBP7 and TIMP-2 are approved by the U.S. Food and Drug Administration as urinary biomarkers for prediction of kidney function. However, the potentially added value of these markers in the circulation in systemic diseases is still largely unknown.

Prevention of injury in kidney transplant recipients

In order to improve the prognosis of kidney function, prevention of kidney injury formation is of key importance. In kidney transplantation, adequate screening of the immunological risk before transplantation is one of the strategies to prevent events that induce kidney injury, such as rejection. As described above, ABMR is characterized by the presence of DSAs and is accompanied by severe kidney injury and impaired long term kidney graft function. A large proportion of these patients are immunized before transplantation and have preformed DSAs present before transplantation. A more sensitive screening method may identify high risk patients better and can therefor guide to alternatives in these patients, such as cross-over transplantations, lower the risk for rejection, and subsequently prevent kidney injury.

Novel therapeutics after renal transplantation

Next to improved screening methods, prevention of kidney injury may be achieved by improved immunosuppressive treatment of renal recipients. As previously mentioned, immunosuppressive agents, such as tacrolimus, can induce kidney injury. However, CNI withdrawal translates in higher rejection rates.[44] In this context, mesenchymal stromal cell

(MSC) therapy may be an interesting approach to reduce the load of traditional immunosuppressives, because of the presumed immune regulatory response of this cellular therapy.[45-47] MSC's are a heterogeneous population of multipotent cells, that can be obtained from the bone marrow, umbilical cord or adipose tissue. MSCs can condition the immune system, that can lead to self-sustaining tolerogenic activity. Currently, studies in the field of solid organ transplantation are predominantly phase I trials and frequency and dosage of administration are variable between studies.[48] The implementation of MSC therapy in renal recipients may act as an alternative for CNI use and lower the amount of kidney injury, caused by the immunosuppressive regimen.

Outline of this thesis

This thesis studies the development of injury in native kidneys, kidney grafts and the accompanied vascular injury. Mechanisms of cellular processes involved in kidney injury may clarify the pathophysiology and can offer possibilities to useful diagnostic strategies, as well as therapeutic approaches. In addition, optimization of diagnostic strategies may further improve the prognosis and prevent the need for more advanced treatment.

In **chapter 2**, the circulating and urinary levels of the cell cycle biomarkers IGFBP7 and TIMP-2 are assessed in the context of diabetic nephropathy and SPKT. In addition, a subpopulation was followed longitudinally after SPKT.

Chapter 3 describes four vascular-specific circulating lncRNAs in patients with diabetic nephropathy. LncRNAs were correlated with the vascular markers angiopoietin-2 (Ang-2) and soluble thrombomodulin (sTM). Patients with diabetic nephropathy were followed longitudinally after receiving an SPKT.

In **chapter 4**, vascular-specific lncRNAs are determined in kidney transplant recipients with acute rejection and with a stable kidney function. Patients with acute rejection were followed longitudinally and the correlation was assessed between the mentioned vascular-specific lncRNAs and vascular markers Ang-2 and sTM.

Chapter 5 describes an observational cohort study of living unrelated kidney transplant recipients. The incidence of early acute ABMR was assessed and possible risk factors for ABMR are analyzed. A group of patients that have a high risk of developing ABMR (i.e. female recipients who receive a kidney from their male spouse) are investigated in detail and the current pre-transplant screening for preformed donor-specific antibodies in the context of ABMR risk is studied.

In **chapter 6**, MSC therapy is presented as an interesting alternative approach to induce immune suppression, in order to reduce kidney injury. MSC therapy was offered to kidney transplant recipients as a substitute for the calcineurin inhibitor tacrolimus.

Lastly, **chapter 7** discusses the research presented in this thesis and places the conclusions in the broader context of kidney injury and kidney transplantation. A Dutch summary of this thesis is presented in **chapter 8**, next to a curriculum vitae of the author, a list of publications, and dankwoord.

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Chapter 2

Serum TIMP-2, but not IGFBP7, levels remain high despite successful simultaneous pancreas-kidney transplantation

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Submitted

Abstract

Background

Insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases 2 (TIMP-2), both involved in the G1 cell cycle arrest, have mainly been described as urinary biomarkers in the context of acute kidney injury. Elevated serum levels, however, have also been reported in patients with diabetes mellitus (DM) with impaired kidney function. Differentiation between kidney injury and systemic vascular damage may be difficult, especially in the early stages of diabetic nephropathy. The objective of this study was to assess urinary and serum IGFBP7 and TIMP-2 levels in type-1 diabetics with either preserved renal function or advanced renal dysfunction (i.e. eGFR ≤30ml/min/1.73m^2), as well as recipients of a successful kidney (KTA) or simultaneous pancreas-kidney transplant (SPKT). SPKT recipients were followed longitudinally during the first year after transplantation to clarify the course of these biomarkers in case of replacement of both kidney and endogenous pancreas function.

Methods

Serum and urinary IGFBP7 and TIMP-2 concentrations were measured using ELISA assays, in 96 individuals; patients with type-1 DM with an eGFR >30ml/min/1.73m^2 (DM>30; n=13), DM patients with an eGFR ≤30ml/min/1.73m^2 (DM≤30; n=17), healthy controls (HC; n=14), and recipients of a KTA (n=14) or SPKT (n=36). DM≤30 patients who received a SPKT (n=18) were followed at 1, 6 and 12 months after transplantation.

Results

Circulating IGFBP7 and TIMP-2 were significantly higher in DM≤30 as compared to HC. In addition, both circulating IGFBP7 and TIMP-2 decreased rapidly after a successful SPKT and IGFBP7 remained low during follow-up. The serum IGFBP7 level was highly dependent upon glomerular filtration. In contrast, despite adequate kidney graft function, circulating TIMP-2 levels returned within the first year to levels comparable to those found in patients with DM>30 or after KTA. In addition, TIMP-2 correlated significantly with the vascular injury marker angiopoietin-2. Urinary levels of either IGFBP7 or TIMP-2 showed a high variation, but did not differ significantly between the different groups.

Conclusions

Circulating IGFBP7 and TIMP-2 levels were higher in type-1 diabetics with impaired renal function. While increased IGFBP7 levels were associated with glomerular filtration, TIMP-2 levels remained significantly higher in type-1 diabetics after a successful SPKT, suggesting persistent and chronic vascular injury.

Introduction

Insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases 2 (TIMP-2) are both biomarkers involved in G1 cell cycle arrest and initially described as urinary biomarkers in the context of acute kidney injury.[1-3] Because of the reported predictive value of IGFBP7 and TIMP-2 for clinical acute kidney injury prediction,[4,5] the U.S. Food and Drug Administration approved these markers for this purpose. Next to acute kidney injury, increased IGFBP7 and/or TIMP-2 levels have been reported in the context of systemic (vascular) diseases, such as diabetes mellitus (DM),[6,7] chronic kidney injury and chronic allograft injury after renal transplantation.[8-10] The assumption of a role in the pathophysiology of vascular disease is further supported by the association of IGFBP7 and TIMP-2 with non-renal diseases, such as malignancies[11,12] and endometriosis.[13] The association between IGFBP7 and TIMP-2 is also of interest in relation to microvascular complications and evolution of diabetic nephropathy (DN). The prognosis of type-1 diabetes is highly dependent on the progression of microvascular complications, such as neuropathy, retinopathy and nephropathy. Diabetic nephropathy is a common vascular complication in type-1 diabetes[14] and may lead to end-stage renal disease in a relevant proportion of patients. The primary goal of treatment in patients with diabetic nephropathy is preservation of kidney function by optimal glycemic control and reduction of albuminuria by adequate blood pressure regulation, in order to prevent or at least delay the need for renal replacement therapy.[15] Progression to end-stage renal disease is however diverse and difficult to predict for individual patients with diabetic nephropathy. More knowledge is needed about the pathophysiology to clarify this phenomenon and biomarkers are of great importance to identify patients in an early stage of DN.

In case of progression to end-stage renal disease, a simultaneous pancreas kidney transplant (SPKT) is the preferred treatment option that replaces kidney function and also restores endogenous insulin secretion. Changes over time of serum TIMP-2 and IGFBP7 levels after SPKT and a kidney transplant alone (KTA) are unclear, but may provide relevant information on the etiology of the altered IGFBP7 and TIMP-2 levels observed in diabetic nephropathy and potentially differentiate between a change in glomerular filtration and/or ongoing systemic (micro)vascular disease. The aim of this study was to assess urinary and serum IGFBP7 and TIMP-2 levels in the context of type-1 diabetes with and without end-stage renal disease. Subsequently, changes were studied in type-1 DM patients after (pancreas) kidney transplantation.

Materials and methods

Study cohort

This single-center, cross-sectional study consists of 96 individuals enrolled at the Leiden University Medical Center (LUMC) with a one-year follow-up in the subgroup who received a combined pancreas-kidney transplant. The cross-sectional cohort consisted of five groups; patients with type-1 DM with preserved renal function (estimated glomerular filtration rate (eGFR) >30ml/min/1.73m^2; DM>30: n=13), type-1 DM patients with impaired renal function (eGFR ≤30ml/min/1.73m^2; DM≤30: n=17), healthy controls (HC: n=14), and type-1 DM patients with end-stage renal disease who received a SPKT (n=36) or KTA (n=14). The KTA group consisted of type-1 DM patients not suitable for SPKT (n=11) or those with an early failed pancreas graft due to vascular thrombosis within four days after transplantation (n=3). The DM≤30 patients from the cross-sectional study who received an SPKT had a follow-up of 1, 6 and 12 months after transplantation. Baseline characteristics of the cross-sectional study, as well as the clinical follow up characteristics of the SPKT subgroup (body mass index, blood pressure, HbA_{1c}, glucose, eGFR, and proteinuria), were retrieved from the electronic health records. All SPKT and KTA patients were transplanted at the LUMC between 1991 and 2012 and received the immunosuppressive regime according to the protocol at the time of transplantation, as previously described.[16,17]

The study design was approved by the Medical Ethical Committee of the LUMC. Written informed consent was obtained from all participants.

Assessment of urinary and serum TIMP-2 and IGFBP7 levels

Blood and urinary samples from all participants were obtained in the outpatient clinic and directly centrifuged and stored at -80°C. Urinary and serum TIMP-2 and IGFBP7 were quantified using sandwich enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (ELISA, Cat. Nr. DTM200, R&D systems, Minneapolis, MN for TIMP-2, and Cat. Nr. EK0991, Boster Biological Technology, Pleasanton, CA for IGFBP7, respectively). Concentrations of urinary and serum TIMP-2 and IGFBP7 were within linear range after sample dilution. Analysis for TIMP-2 and IGFBP7 was performed with one reagent lot number. Low and high-level quality control (IQC) urine samples were prepared from pooled urine by spiking and analyzed in triplicate on each sample plate to assess the stability of the assay. The mean analytical imprecision (expressed as CV%) for serum TIMP-2 was 2.8% (at 3292 pmol/l). For urinary TIMP-2, analytical imprecision was 4.1% (at 183 pmol/l) for low IQC and 4.4% (at 239 pmol/l) for high IQC. For urinary IGFBP7, CV% was 11.5% (at 831 pmol/l) for low IQC and 9.1% (at 2141 pmol/l) for high IQC. Creatinine and total protein were measured using a Cobas c502 analyzer (Roche Diagnostics, Mannheim, DE), according to the manufacturer's instructions. The vascular marker angiopoietin-2 (Ang-

2), previously determined in this cross-sectional cohort,[16,18] was included in the analysis of this study as marker for vascular injury.

Statistical analyses

Parametric data are described as mean ± SD, non-parametric data as median and IQR, and categorical data as numbers and percentages. Baseline characteristics were tested for differences using one-way ANOVA (parametric data), Kruskall-Wallis (non-parametric data), and Fisher Exact test (categorical data). Baseline characteristics of the longitudinal cohort were tested for differences using the paired T-test (parametric data), Wilcoxon signed-rank test (non-parametric data) and Friedman's two-way ANOVA by ranks (categorical data).

Urinary levels were corrected for urinary creatinine from the same sample. Circulating and urinary IGFBP7 and TIMP-2 levels were both reported as logarithmic levels and herewith showed a normal distribution. Fractional excretion of IGFBP7 and TIMP-2 were calculated by ([urinary biomarker] x [serum creatinine] / [serum biomarker] x [urinary creatinine]) and showed a normal distribution after logarithmic transformation. Cross-sectional results were analyzed with a univariate general linear model. Longitudinal results were analyzed using a linear-mixed model analysis. Correlations were analyzed using a Spearman rank correlation.

A value of $p < 0.05$ was considered as statistically significant. Data analysis was performed with SPSS version 23.0 (SPSS Inc., Chicago IL, USA) and creation of graphs with GraphPad Prism version 8.0 (Graphpad Prism Software Inc., San Diego, CA, USA).

Results

Patient characteristics

Baseline characteristics of the participants are summarized in *Table 1*. The median time since the diagnosis of type-1 DM in this cohort was 30 years (IQR 22-38 years) and comparable in patients with DM≤30 and SPKT recipients. After a successful SPKT or KTA the mean eGFR was significantly better as compared to DM≤30 patients (both <0.001). The mean HbA_{1c} was normal and significantly lower in the SPKT recipients as compared with all other subgroups of diabetics. The organs of all SPKT and 36% of KTA originated from deceased donors. All SPKT and 79% of KTA patients received a calcineurin inhibitor (tacrolimus or cyclosporin) and prednisone use was 69% and 64% in the SPKT and KTA group respectively. The type-1 DM patients in the longitudinal study had adequate glucose regulation after successful SPKT (HbA_{1c} 37.7 ± 8.7 and glucose levels 5.8 ± 2.8) and mean eGFR of their kidney was 54 ± 12 one year after transplantation (*Table 2*). In addition, these SPKT patients had a mean blood pressure of 128/77 one year after transplantation and minimal proteinuria. Patient and graft (both kidney and pancreas) survival were 100% in the study period.

Table 1. *Cross-sectional study patient characteristics.*

Characteristics	HC (n=14)	DM>30 (n=13)	KTA (n=14)	DM≤30 (n=17)	SPKT (n=36)
Sex, *male, n (%)*	7 (50%)	6 (46%)	6 (43%)	13 (77%)	23 (64%)
Age *(years)*	48 ± 11	52 ± 14	48 ± 10	45 ± 6	48 ± 8
BMI *(kg/m^2)*	25.0 ± 3.7	23.9 ± 2.5	25.2 ± 4.8	24.9 ± 3.4	24.3 ± 4.4
Systolic BP *(mmHg)*	132 ± 14	129 ± 11	136 ± 30	147 ± 18	139 ± 23
Diastolic BP *(mmHg)*	83 ± 7	72 ± 10	80 ± 14	85 ± 9	83 ± 13
Smoking, *n (%)*	0	2 (15%)	1 (7%)	0	3 (8%)
Duration of diabetes *(y)*	-	34 + 10	36 ± 9	28 ± 9	27 ± 8
Time since Tx *(months) median (IQR)*	-	-	25 (10-65)	-	45 (19-107)
HbA$_{1c}$ *(mmol/mol)*	-	57 ± 13	70 ± 10	74 ± 20	38 ± 9
Glucose *(mmol/l)*	5.3 ± 0.9	12.6 ± 4.9	13.6 ± 6.6	12.9 ± 6.6	5.8 ± 2.8
eGFR *(ml/min/1.73m^2)*	91 ± 14	72 ± 24	64 ± 23	18 ± 7	53 ± 20
Proteinuria *(g/24 h) median (IQR)*	-	0.26 (0.18-0.41)	0.21 (0.18-0.23)	0.75 (0.54-1.30)	0.28 (0.19-0.82)

HC = healthy controls, DM>30 = diabetes mellitus with an eGFR >30ml/min/1.73m^2, DM≤30 = diabetes mellitus with an eGFR ≤30 ml/min/1.73m^2, SPKT = simultaneous pancreas-kidney transplantation, KTA = kidney transplantation alone, BMI = body mass index.

Table 2. *Longitudinal study patient characteristics of type-1 diabetes patients with an eGFR ≤30 ml/min/1.73m^2, who received a simultaneous pancreas-kidney transplantation (n=18).*

Characteristics	D0 (n=14)	M1 (n=12)	M6 (n=14)	M12 (n=12)
Systolic BP *(mmHg)*	151 ± 17	132 ± 14	131 ± 20	128 ± 19
Diastolic BP *(mmHg)*	86 ± 10	78 ± 9	78 ± 10	77 ± 5
HbA$_{1c}$ *(mmol/mol)*	74 ± 18	45 ± 18	34 ± 3	36 ± 3
Glucose *(mmol/l)*	13.7 ± 6.7	6.5 ± 1.1	5.2 ± 1.2	6.1 ± 1.7
eGFR *(ml/min/1.73m^2)*	17 ± 7	48 ± 17	52 ± 13	54 ± 12
Proteinuria *(g/24 h) median (IQR)*	0.75 (0.67-1.29)	0.63 (0.27-1.05)	0.34 (0.25-0.98)	0.26 (0.12-0.50)

D0 = before transplantation, M1 = 1 month after transplantation, M6 = 6 months after transplantation, M12 = 12 months after transplantation, BP = blood pressure, BMI = body mass index.

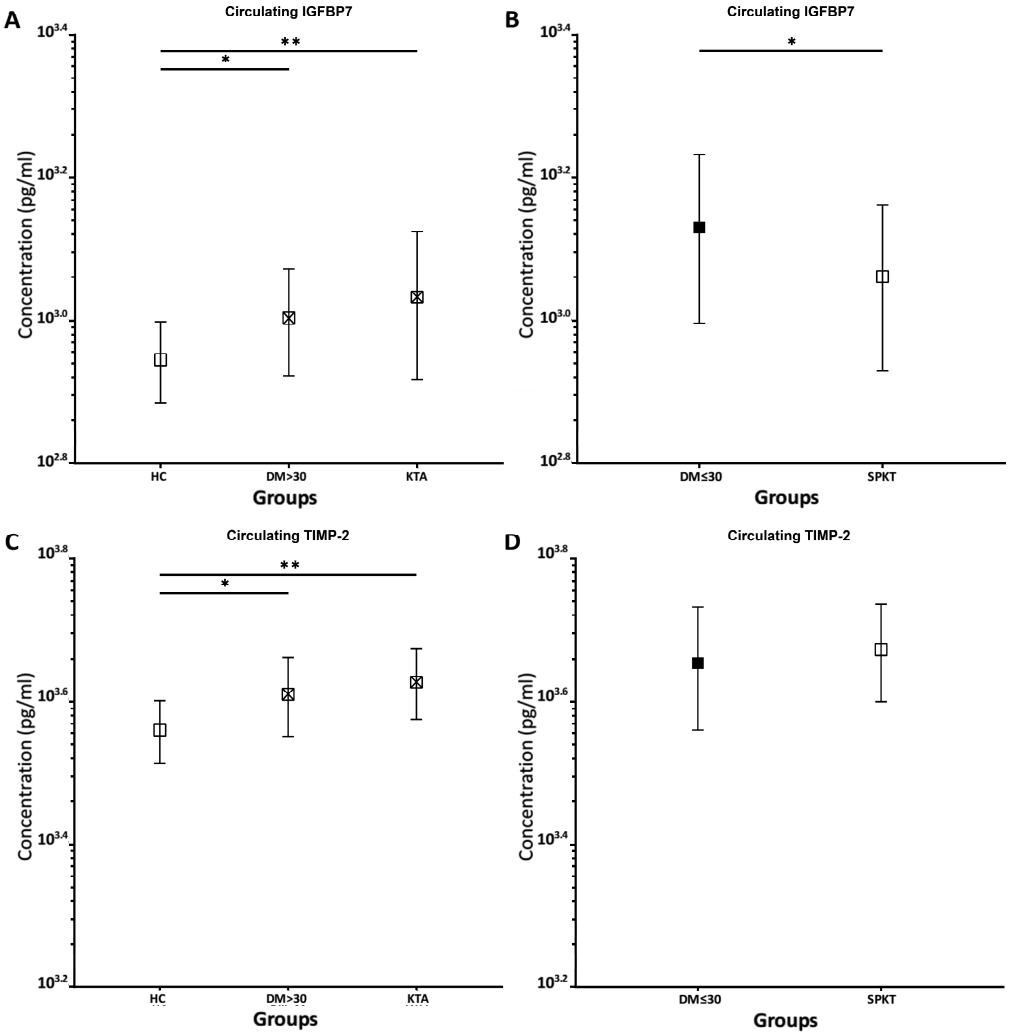

Figure 1. Circulating IGFBP7 and TIMP-2 levels are differently affected by changes in kidney function. *Circulating IGFBP7 (A) and TIMP-2 (C) levels are higher in type-1 DM patients with an eGFR >30ml/min/1.73m^2 (DM>30) or after a successful kidney transplantation alone (KTA), compared with healthy controls (HC). High IGFBP7 levels in type-1 DM patients with an eGFR ≤30ml/min/1.73m^2 (DM≤30) (B) decrease after a simultaneous pancreas-kidney transplantation (SPKT). This is in contrast with TIMP-2 levels (D) that remain high in SPKT patients. * p < 0.05, ** p < 0.01.*

Type-1 diabetes and circulating IGFBP7 and TIMP-2 concentrations

Patients with type-1 diabetes and an eGFR >30ml/min/1.73m^2 (DM>30) had significantly higher circulating IGFBP7 (p=0.04) and TIMP-2 (p=0.02) levels, as compared to healthy controls (*Figure 1*). In case of an eGFR ≤30ml/min/1.73m^2 (DM≤30), the IGFBP7 concentration was significantly higher (p=0.003) as compared to DM>30, while TIMP-2 levels were comparable (p=0.18). Type-1 diabetics, who received a successful KTA, had similar levels of IGFBP7 and TIMP-2, as found in type-1 DM patients with eGFR >30ml/min/1.73m^2. Urinary IGFBP7 and TIMP-2 levels, either corrected for urinary creatinine concentration or expressed as fractional excretion in relation to creatinine, showed a wide variation but no statistically significant differences between the different groups in this cohort with an eGFR >30ml/min/1.73m^2 (*Supplementary Figure 1*). In addition, no significant correlations between urinary IGFBP7 or TIMP-2 levels with the corresponding circulating levels were found.

Only serum IGFBP7 decreased after simultaneous pancreas-kidney transplantation

In order to further differentiate between impaired glomerular filtration and ongoing systemic vascular disease in DM patients, samples of DM≤30 patients and DM patients who received an SPKT were analyzed. Circulating IGFBP7 decreased after a successful SPKT transplant, compared with DM≤30 (p=0.03). TIMP-2, however, remained at higher levels (p=0.07), despite having adequate kidney transplant function and restored endogenous insulin secretion.

Dynamics of TIMP-2 and IGFBP7 after a simultaneous pancreas kidney transplantation

Follow-up of DM patients who received an SPKT in the first year after transplantation showed IGFBP7 to decline rapidly and remain significantly lower (p<0.001), while TIMP-2 levels, only temporarily declined after the successful SPKT (p<0.001) with a gradual return towards pre-transplant levels (*Figure 2*). Theoretically this could be explained by the gradual lowering of immunosuppressive drugs, (return of) chronic systemic inflammation, or presence of chronic vascular injury. To test this hypothesis, we evaluated the correlation of circulating IGFBP7 and TIMP-2 with the vascular biomarker Ang-2. TIMP-2 correlated significantly with Ang-2 (r=0.25, p=0.021), while IGFBP7 was not significantly correlated (r=0.15; p=0.18).

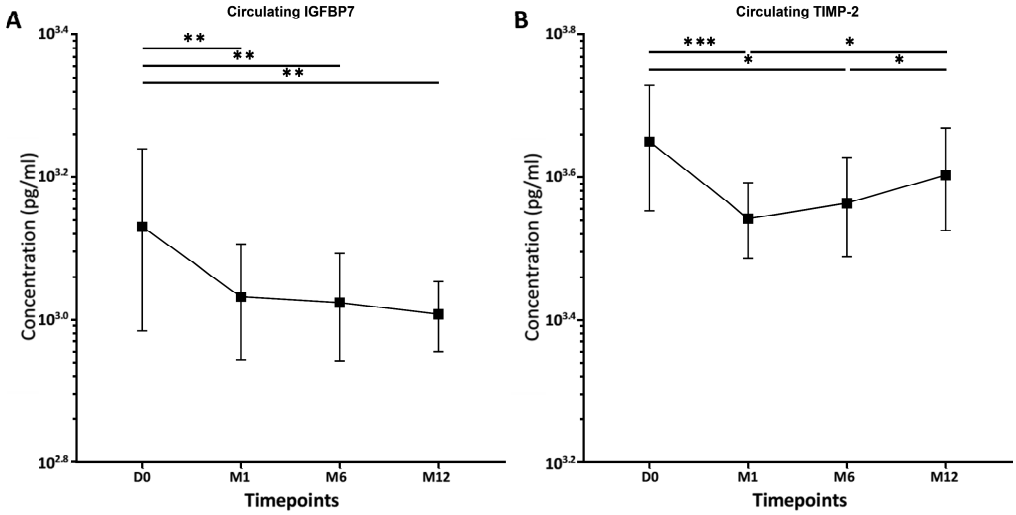

Figure 2. Circulating IGFBP7 (A) and TIMP-2 (B) have a different course after simultaneous pancreas-kidney transplantation. *IGFBP7 and TIMP-2 both decline shortly after simultaneous pancreas-kidney transplantation. Subsequently, IGFBP7 remains lower while TIMP-2 increased one year after transplantation. Levels are depicted as logarithmic values (mean ± SD). D0 = before transplantation, M1 = 1 month after transplantation, M6 = 6 months after transplantation, M12 = 12 months after transplantation. * p < 0.05, ** p < 0.01, *** p < 0.001.*

Discussion

This study shows that circulating levels of IGFBP7 and TIMP-2 are significantly higher in type-1 DM patients, compared with healthy controls. In type-1 DM patients with impaired kidney function (DM≤30), circulating levels of IGFBP7 further increased, most likely due to the fact that higher IGFBP7 is predominantly associated with impaired kidney function. This is supported by lower levels of IGFBP7 in SPKT recipients and the rapid persistent decline of IGFBP7 in the first year after a successful SPKT. TIMP-2, interestingly, does not significantly differ between DM≤30 patients and SPKT recipients. This is in accordance with the gradual return to pretransplant levels of TIMP-2 after SPKT. Therefore, TIMP-2 is suggested to be associated with chronic vascular injury, next to glomerular filtration.

Interestingly, circulating TIMP-2 remained high in type-1 DM patients who received a SPKT, although TIMP-2 did have a weak correlation with eGFR. High circulating TIMP-2 levels in DM may therefore be the result of diabetes related systemic factors, next to altered glomerular filtration. As shown in *Supplementary Figure 1*, the fractional excretion of TIMP-2 was comparable between transplanted DM patients (KTA and SPKT) and HC and we did not find a correlation between creatinine corrected urinary levels and circulating levels of IGFBP7 and TIMP-2. This suggests that the higher levels of TIMP-2 in transplanted patients are not due to altered excretion, but may be the consequence of increased systemic

production and/or altered systemic dynamics and metabolism. The longitudinal study supports this hypothesis and shows similar circulating TIMP-2 levels one year after transplantation, compared with end-stage renal disease. Previously, TIMP-2 is described to be higher in chronic vascular injury. Higher levels of TIMP-2 have also been shown to correlate with interstitial fibrosis in patients with chronic allograft damage.[19] In contrast with circulating levels, urinary levels did not demonstrate differences between the groups in the cohort. Next to systemic production of IGFBP7 and TIMP-2, urinary levels are also highly dependent upon differences in renal excretion. A recent study showed urinary IGFBP7 and TIMP-2 to increase, due to increased filtration, decreased tubular reabsorption and leakage of IGFBP7 and TIMP-2 in the proximal tubule.[20] In addition, urinary levels may change due to higher suggested TIMP-2 levels locally in the kidney.[21]

Next to altered levels of TIMP-2 due to diabetes and kidney function, TIMP-2 may be higher in transplant recipients in general. Previous research showed kidney transplant recipients to have higher TIMP-2 levels in plasma, compared with healthy controls.[22] This is in contrast with our data, since we observed comparable levels of IGFBP7 and TIMP-2 between DM>30 patients and type-1 DM patients, who received a kidney transplantation alone. Different immunosuppressive regimes within the SPKT and KTA group did not show a correlation with IGFBP7 and TIMP-2 levels. Additionally, Bicknell showed no difference in TIMP-2 levels between patients that received tacrolimus or ciclosporin.[23]

The specific role of IGFBP7 and TIMP-2 in the etiology of the different types of kidney damage remains a subject of discussion. Both IGFBP7 and TIMP-2 are often described as indicators of kidney damage. However, it is increasingly recognized that IGFBP7 and TIMP-2 also play an active role in the etiology of kidney damage. TIMP-2 is considered to play a role in both the occurrence and the progression of renal lesions in DM patients.[24] IGFBP7 may serve as a factor in TGF-β1-induced tubular injury in DN.[25] In addition, IGFBP7 is suggested to play a role in hyperglycemia-related podocyte proliferation, by the TGF-β1/Smad pathway.[26] This is an interesting finding, since we found levels of circulating IGFBP7 and TIMP-2 to be elevated in DM with a preserved kidney function. Therefore IGFBP7 and TIMP-2 may be particularly interesting biomarkers in the context of this early stage of damage in DM patients. It would be interesting to investigate IGFBP7 and TIMP-2 levels in an even earlier stage of DM with micro-albuminuria to assess the added value of IGFBP7 and TIMP-2 in the early diagnosis of DM and prognosis of kidney function in these patients.

The main strength of this study is the assessment of both circulating and urinary levels in a cross-sectional and longitudinal cohort. This approach facilitates a better differentiation between kidney function related changes and the association with other factors, such as chronic vascular injury. Additionally, the KTA group acts as a control group for the SPKT group and differentiates between the effect of improved kidney function and improved glucose regulation.

This study has several limitations. Although the cohort is a well-defined cohort, group size was limited. In addition, the influence of impaired kidney function in transplant recipients could not be observed. Therefore, a group of KTA or SPKT patients with deteriorated kidney function would be interesting to study as well.

Conclusions

Circulating IGFBP7 and TIMP-2 are significantly higher in patients with DM. IGFBP7 shows to be largely dependent upon glomerular filtration and consequently return to normal levels, after a successful kidney transplantation. In contrast, circulating TIMP-2 levels remain higher, despite kidney transplantation and restoration of endogenous insulin secretion, suggesting a role in ongoing chronic and/or persistent vascular injury.

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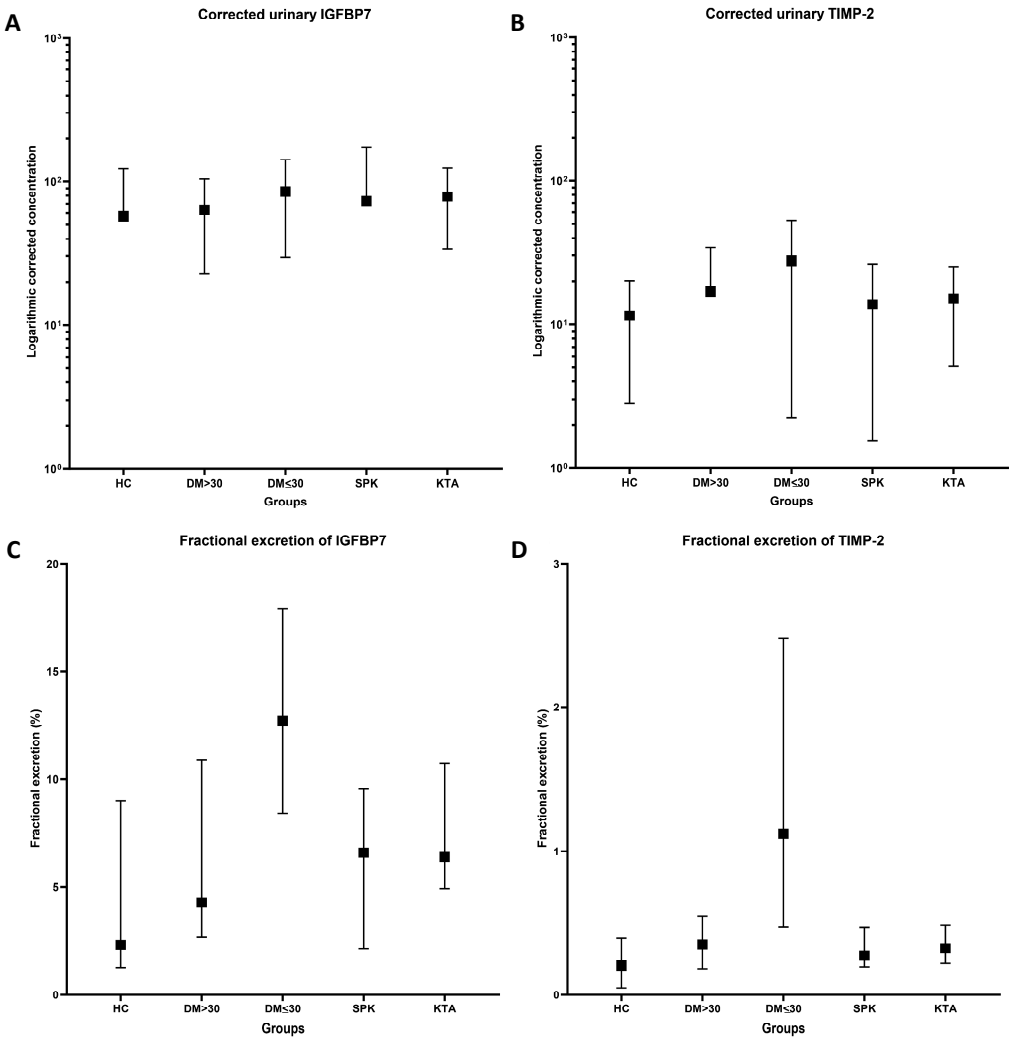
Supplementary information



Supplementary Figure S1. Urinary levels and fractional excretion of IGFBP7 (A and C) and TIMP-2 (B and D). *Urinary levels of IGFBP7 and TIMP-2 are comparable between the groups in the cohort. Fractional excretion of TIMP-2 was comparable between healthy controls (HC), type-1 DM patients with an eGFR >30ml/min/1.73m^2 (DM>30), and type-1 diabetes patients who received a simultaneous pancreas-kidney transplantation (SPKT) or a kidney transplantation alone (KTA).*

Chapter 3

Diabetic nephropathy alters circulating long noncoding RNA levels that normalize following simultaneous pancreas-kidney transplantation

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Abstract

Background

Simultaneous pancreas-kidney transplantation (SPKT) replaces kidney function and restores endogenous insulin secretion in patients with diabetic nephropathy (DN). Here, we aimed to identify circulating long noncoding RNAs (lncRNAs) that are associated with DN and vascular injury in the context of SPKT.

Methods

Based on a pilot study and a literature-based selection of vascular injury-related lncRNAs, we assessed 9 candidate lncRNAs in plasma samples of patients with diabetes mellitus with a kidney function >35 mL/min/1.73 m^2 (DM; n=12), DN (n=14), SPKT (n=35), healthy controls (n=15), and renal transplant recipients (KTx; n=13). DN patients were also studied longitudinally before and 1, 6, and 12 months after SPKT.

Results

Of 9 selected lncRNAs, we found MALAT1, LIPCAR, and LNC-EPHA6 to be higher in DN compared with healthy controls. SPKT caused MALAT1, LIPCAR, and LNC-EPHA6 to normalize to levels of healthy controls, which was confirmed in the longitudinal study. In addition, we observed a strong association between MALAT1, LNC-EPHA6, and LIPCAR and vascular injury marker soluble thrombomodulin and a subset of angiogenic microRNAs (miR-27a, miR-130b, miR-152, and miR-340).

Conclusions

We conclude that specific circulating lncRNAs associate with DN-related vascular injury and normalize after SPKT, suggesting that lncRNAs may provide a promising novel monitoring strategy for vascular integrity in the context of SPKT.

Introduction

Diabetes mellitus (DM) is a major cause of end-stage renal disease and leads to microvascular complications such as retinopathy and neuropathy.[1-3] Because diabetic nephropathy (DN) is characterized by albuminuria and elevated blood pressure, the main early goals in preservation of kidney function, in addition to preventing hyperglycemia, are reducing microalbuminuria and hypertension.[4] Ultimately, when end-stage renal disease develops, simultaneous pancreas-kidney transplantation (SPKT) is a preferred treatment option that replaces kidney function and restores endogenous insulin secretion in patients with DN.

However, integrity of the vasculature is a rate-limiting factor in the long-term outcome of organ transplants.[5] Although the endothelial dysfunction associated with DN is partly restored after transplantation,[6-8] the endothelium in SPKT is further challenged by transplant-specific adverse effects such as ischemia-reperfusion injury and following the use of immunosuppressive drugs, such as steroids and calcineurin inhibitors, that exhibit unfavorable effects on the vasculature. In addition, viral infections or acute rejection are known to affect microvascular integrity.[9-11] Taken together, due to these risk factors, the vasculature is continually challenged. Thus, to preserve graft function, monitoring of microvascular integrity may be of high clinical value as patients could receive targeted treatment.

We previously demonstrated that SPKT reversed microvascular damage in DN[8] and found that specific microRNAs (miRNAs) are associated with DN and microvascular impairment and vascular injury markers, such as angiopoietin-2 (Ang-2) and soluble thrombomodulin (sTM).[7,12] Recently, long noncoding RNAs (lncRNAs) have been recognized as important regulators of gene expression and may be promising candidate biomarkers for early recognition of disease progression.[13] LncRNAs are defined as noncoding transcripts longer than 200 nucleotides, interfere with a variety of cellular processes, and are involved in the pathophysiology of a broad range of diseases including kidney and vascular diseases.[14,15] For example, increased levels of the lncRNA MALAT1 have been described to associate with DM and the development of organ dysfunction, such as retinopathy and nephropathy, by contributing to inflammation and the impaired response of endothelial cells to glucose.[16] Also, the long noncoding megacluster (lnc-MGC), hosting a cluster of nearly 40 miRNAs has been described to be involved in the development of diabetic kidney disease, most likely via endothelium reticulum stress-dependent mechanisms.[17] However, although an initial study demonstrated differences in circulating lncRNA levels in DN patients compared with healthy controls,[18] little is known about the relation of circulating lncRNAs with DN and vascular injury, in particular in the unique context of SPKT.

The aim of this study was to identify lncRNAs that are associated with SPKT and (micro)vascular injury. This could provide more insight in the development of vascular

complications and may identify specific lncRNAs to be of benefit for predicting or combating vascular injury progression.

Materials and methods

Study cohort

Study design and all study procedures were approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC), and written informed consent was obtained from all participants.

In a single-center, cross-sectional, observational study, 78 individuals aged 18 years or older were enrolled. Four groups of patients with DM type 1, all treated in the outpatient clinic of the LUMC, were included: a group of DM patients with signs of early DN (eGFR >35 mL/min/1.73 m^2) (DM; n=12), a group of DM patients with DN on the waiting list for SPKT (DN; n=14), a group of DM patients with functioning pancreas and kidney grafts (SPKT; n=35), and a group of DM patients with a functioning kidney graft (KTx; n=13) consisting of 10 patients with a solitary kidney transplant and 3 patients who initially received an SPKT but lost their pancreatic graft within 4 days after transplantation due to vascular thrombosis. A control group consisted of 15 healthy, age-matched volunteers. Only patients with a sufficient amount of plasma for all required assays were included in this study. Exclusion criteria were active infection or autoimmune disease, liver failure, epilepsy, and malignancy in the past 5 years (excepted full remission after treatment for basal cell carcinoma).

This cohort was previously described and was studied for a selection of circulating miRNAs for microvascular endothelial injury, sTM, and angiopoietin-2 (Ang-2) in plasma samples of all participants.[8]

Sixteen DN patients who received an SPKT were followed longitudinally during the first year after transplantation. Plasma samples of these patients were obtained before and 1, 6, and 12 months after transplantation, but plasma samples for all 4 timepoints were not available for all 16 patients. The available group size for each timepoint is shown in *Table 2*.

Identification of candidate lncRNAs

To identify candidate lncRNAs, we performed a pilot study and a literature-based selection. For the pilot study, we selected candidate lncRNAs by assessing plasma profiles of 40,173 lncRNAs in 6 randomly selected healthy controls and 6 DN patients. LncRNAs were selected based on differential expression ($P < 0.001$ or a fold change >50 combined with a value of $P < 0.05$). Second, we performed a literature search to select a set of candidate lncRNAs that have been described to associate with vascular injury (described in detail in Results). Together, this resulted in the selection of 22 lncRNAs. Using RT-qPCR validation, only 9 of these 22 lncRNAs were detectable and assessed in the whole patient cohort. To ensure

robust expression, only lncRNAs with >95% of the samples showing detectable expression were selected for further analysis, yielding 4 lncRNAs: LNC-EPHA6, MALAT1, LIPCAR, and LNC-RPS24.

Transplantation and follow-up

All vital parameters and blood and urine samples were measured and collected at the outpatient clinic of the LUMC. Both KTx and SPKT were performed in the LUMC, and these procedures were described previously.[8,19] Frequent follow-up of transplanted patients took place at the transplantation outpatient clinic in the LUMC.

All SPKT patients and 86% of KTx patients were treated with calcineurin inhibitors (65% tacrolimus, 35% cyclosporine). Prednisone use in SPKT and KTx was 70% and 60%, respectively. Most SPKT and KTx patients were treated with triple therapy including mycophenolate mofetil (73% and 93%, respectively).

RNA isolation

By using the RNeasy Micro Kit (Qiagen) with an adapted protocol, total RNA was isolated from 200 µL plasma using 800 µL TRIzol reagent (Invitrogen). In summary, the plasma/TRIzol sample was centrifuged for 15 minutes (15,000g) after the addition of 160 µL chloroform. After the aqueous phase was combined with 100% ethanol (1.5 volume), it was transferred to a MinElute Spin column (Qiagen) and centrifuged for 15 seconds (18,000g). The column was then washed with 700 µL RWT buffer and twice with 500 µL RPE buffer. This was centrifuged for 15 seconds (18,000g) after the first 2 washing steps and 2 minutes (18,000g) after the third washing step. Then, 15 µL RNase-free water was added to elute the RNA.

Profiling lncRNAs

The lncRNA profiling was performed by Arraystar Inc. In brief, for the microarray analysis, the Agilent Array platform was used. Sample preparation and microarray hybridization were performed according to the manufacturer's protocols with some minor modifications. Samples were amplified and transcribed into fluorescent cRNA along the entire length of the transcripts with no 3' bias using a random priming method (Arraystar Flash RNA Labeling Kit; Arraystar). The labeled cRNA was hybridized onto the Human lncRNA Array v4.0 (8 x 60K; Arraystar), containing 40,173 lncRNAs. After washing of the slides, the arrays were scanned using the Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used for analysis of the acquired array images. The GeneSpring GX v12.1 software package (Agilent Technologies) was used for quantile normalization and subsequent data processing. After this quantile normalization of the raw data, lncRNAs that have flags in present or marginal ("all targets value"; in at least 6 of 12 samples) were selected for further data analysis. Volcano Plot filtering was used to identify statistically significant differentially expressed lncRNAs

between the two groups. Finally, hierarchical clustering was performed to show distinguishable lncRNAs expression pattern among the groups.

RT-qPCR

For validation of identified lncRNAs, we performed RT-qPCR. To quantify lncRNA levels, isolated RNA was reverse transcribed using Iscript (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR of target genes was done using SYBR Green Master Mix (Applied Biosystems). Used primer sequences of target lncRNAs are given in *Table S1*.

Statistical analyses

All parametric data are described as mean ± SD, and nonparametric data are presented as median and IQR. Categorical variables are given as numbers and percentages. Testing for differences in *Tables 1 and 2* was performed by using 1-way ANOVA for parametric data, Kruskall-Wallis test for nonparametric data, and Fisher exact test for categorical data.

All lncRNA results were normalized by the $CT^{\Delta\Delta}$ method to β-actin, as previously described.[20-23] After logarithmic transformation (with base 10), all lncRNAs showed a normal distribution and were then further analyzed. In the cross-sectional study, differences in logarithmic mean lncRNA levels were analyzed using a univariate general linear model including adjustment for sex and age. For analysis of data in the longitudinal study, a linear-mixed model analysis was used (with inclusion of repeated-measures analysis and adjustment for multiple testing). Categorical data were analyzed for differences using Friedman 2-way ANOVA by ranks. Correlations between vascular markers and lncRNAs were analyzed using the Spearman rank correlation.

A value of $p < 0.05$ was considered to be statistically significant. All data analysis was performed using SPSS version 23.0 (SPSS Inc.), and graphs were created using GraphPad Prism version 8.0 (GraphPad Prism Software Inc.).

Results

Identification of candidate lncRNAs

To identify candidate lncRNAs that are associated with DN, we assessed plasma levels of 40,173 lncRNAs in a pilot study in 6 healthy individuals and 6 DN patients. In addition, we selected a subset of lncRNAs from the literature that were previously described to associate with vascular injury (*Figure 1A* describes our identification strategy). *Figure 1B,C* illustrates a clear differential lncRNA profile in our pilot study in plasma of DN patients compared with healthy controls (full profiling data of this pilot study can be found in *Table S2*). Of 40,173 lncRNAs, 11,517 (29%) were detectable in the microarray analysis: 185 were significantly upregulated and 103 were significantly downregulated ($P < 0.05$).

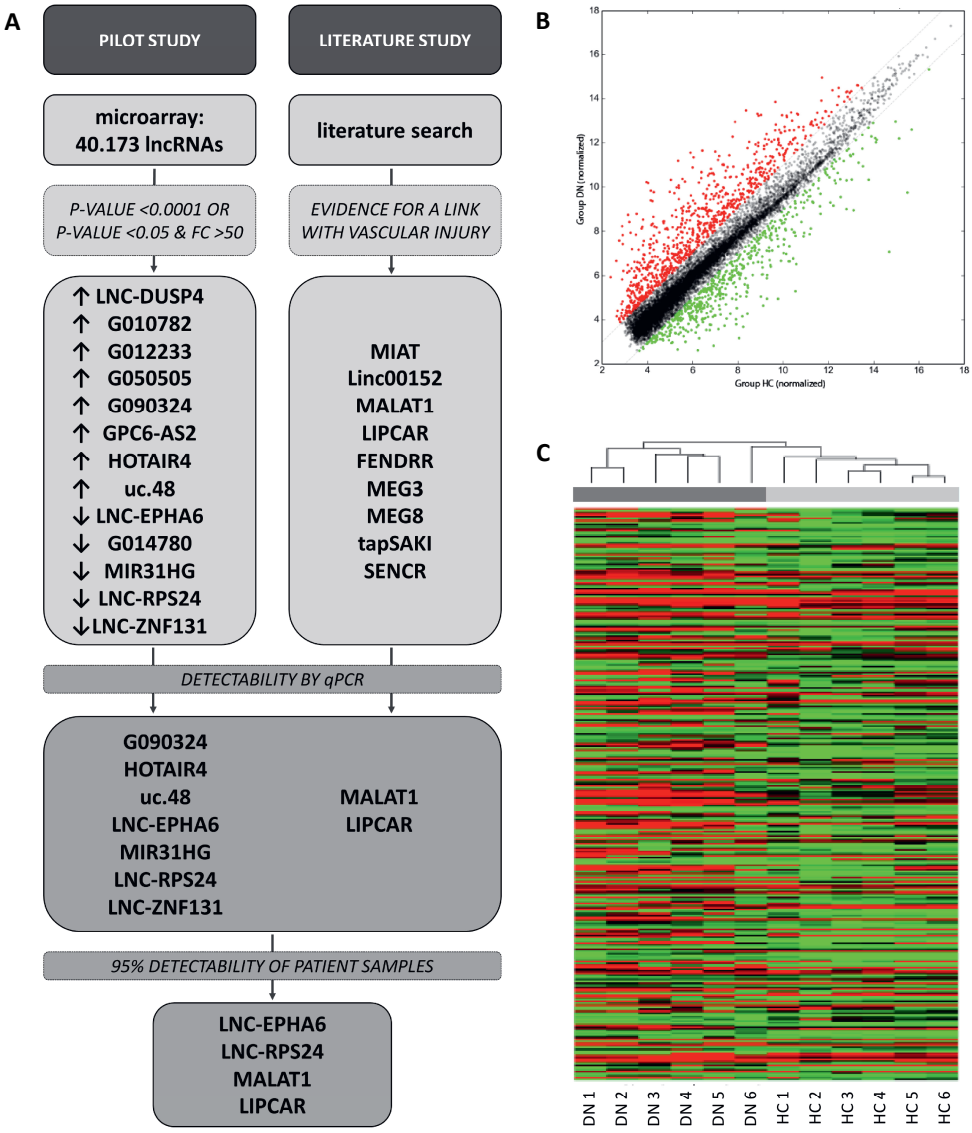

Figure 1. Identification of candidate lncRNAs that associate with diabetic nephropathy. *(A) Schematic overview of identification strategy of candidate lncRNAs, based on a pilot profiling study in plasma of 6 healthy controls (HC) and 6 diabetic nephropathy (DN) patients, as well as a literature-based selection of lncRNAs that have been described to associate with vascular injury. (B) Scatterplot visualizing differential lncRNA expression between indicated conditions. The red and the green points in the plot represents the statistically significant up and down-regulated LncRNAs, respectively, in DN as compared to HC. (C) Hierarchical clustering shows a distinguishable LncRNA expression profiling among patient plasma samples, visualized in a heatmap. Red depicts high expression, green low expression.*

We subsequently selected 13 candidate lncRNAs that were differentially expressed between DN patients and healthy controls, based on either a fold change above 50 combined with P <0.05 or with P <0.001. Of these lncRNAs, DUSP4, G010782, G012233, G050505, G090324, GPC6-AS2, HOTAIR, and uc.48 were upregulated in DN and LNC-EPHA6, G014780, MIR31HG, LNC-RPS24, and ZNF131 were downregulated in DN. In addition, our literature-based selection yielded 9 additional, vascular injury–related lncRNAs: MIAT,[24,25] Linc00152,[26,27] MALAT1,[28,29] LIPCAR,[30,31] FENDRR,[32,33] MEG3,[34,35] MEG8,[24,36] tapSAKI,[37] and SENCR.[38,39] Next, we determined whether these lncRNAs could be validated and detected using RT-qPCR. Nine of the 22 selected lncRNAs (MALAT1, LNCEPHA6, LIPCAR, LNC-RPS24, G090324, HOTAIR, MIR31HG, uc.48, and ZNF131) were detectable using RT-qPCR in the same plasma samples and were selected for analysis in the main cohort.

Table 1. *Cross-sectional study patient characteristics.*

	HC (n=15)	**DM (n=12)**	**DN (n=14)**	**SPKT (n=35)**	**KTx (n=13)**
Sex, *male, n (%)*	8 (53%)	6 (50%)	9 (64%)	23 (66%)	4 (31%)
Age *(years)*	44 ± 10	54 ± 14	44 ± 6[2]	48 ± 8	48 ± 10
BMI *(kg/m^2)*	24.8 ± 3.6	23.7 ± 2.6	24.3 ± 2.8	24.1 ± 4.2	24.6 ± 4.8
Systolic BP *(mmHg)*	133 ± 13	130 ± 14	141 ± 21	140 ± 23	133 ± 25
Diastolic BP *(mmHg)*	83 ± 7	70 ± 9[1]	84 ± 10[2]	84 ± 13[2]	78 ± 12
Haemoglobin *(mmol/l)*	8.8 ± 0.7	8.4 ± 1.3	7.4 ± 0.7[1,2]	8.2 ± 1.1	7.9 ± 1.0
Haematocrit *(l/l)*	0.42 ± .03	0.41 ± .05	0.36 ± .04[1,2]	0.41 ± .05[3]	0.40 ± .04
HbA_{1c} *(mmol/mol)*	-	53.7 ± 8.9	63.6 ± 15.6	37.7 ± 8.7[2]	67.5 ± 8.7[2]
Glucose *(mmol/l)*	5.2 ± 1.1	12.7 ± 5.1[1]	12.5 ± 5.7[1]	5.9 ± 2.9[2,3]	11.2 ± 5.3[1,4]
eGFR *(ml/min/1.73m^2)*	93 ± 17	71 ± 24	26 ± 17[1]	52 ± 19[1,2,3]	60 ± 24[1,3]
Proteinuria *(g/24 h) median (IQR)*	-	0.29 (0.13-0.53)	0.68 (0.31-1.16)	0.27 (0.18-0.81)[3]	0.21 (0.21-0.36)[3]
Smoking, *n (%)*	0 (0%)	2 (17%)	0 (0%)	3 (9%)	1 (8%)
Anti-HT drugs, *n (%)*					
ACE inhibitor	-	6 (50%)	7 (54%)	13 (37%)	6 (46%)
AT2 antagonist	-	3 (25%)	5 (39%)	8 (23%)	0 (0%)[3,4]
Calcium antagonist	-	1 (8%)	6 (46%)[2]	20 (57%)[2]	5 (38%)
Diuretic	-	5 (42%)	6 (46%)	7 (20%)	4 (31%)
Statin, *n (%)*	-	6 (50%)	8 (62%)	24 (69%)	5 (38%)
Duration of DM (y)	-	33 ± 9	31 ± 9	28 ± 9	36 ± 9[4]
Time since Tx *(months) median (IQR)*	-	-	-	45 (19-102)	21 (9-74)
DM after SPK, *n (%)*	-	-	-	3 (9%)	-

HC = healthy controls, DM = diabetes mellitus (eGFR >35ml/min/1.73m^2), DN = diabetic nephropathy, SPKT = simultaneous pancreas kidney transplantation, KTx = kidney transplantation, BMI = body mass index, BP = blood pressure, HT = hypertension, Tx = transplantation (SPKT or KTx). [1] p-value <0.05 versus HC. [2] p-value <0.05 versus DM. [3] p-value <0.05 versus DN. [4] p-value <0.05 versus SPKT.

Patient characteristics

The baseline characteristics of all individuals of the cross-sectional study (HC, DM, DN, SPKT, and KTx; n=89) are presented in *Table 1*. Mean duration of DM in the DM, DN, SPKT, and KTx groups was >27 years. Diabetic retinopathy was described in all patients with DN and 94% of patients with an SPKT. Due to restoration of endogenous insulin production, glucose and HbA_{1c} levels were lower in patients who received an SPKT compared with DN patients or patients who received a solitary KTx. eGFR was significantly higher in SPKT and KTx compared with DN.

Circulating levels of specific lncRNAs associate with DM and DN

To determine the association between DN and circulating lncRNAs, we measured levels of G090324, HOTAIR4, uc.48, LNC-EPHA6, MIR31HG, LNC-RPS24, ZNF131, MALAT1, and LIPCAR using RT-qPCR in plasma samples of all individuals in the cohort. Only 4 of these 9 lncRNAs (MALAT1, LNC-EPHA6, LNC-RPS24, and LIPCAR) met our criteria of being detectable in >95% of the samples (*Figure 1A*) and were selected for further analysis. Circulating levels of MALAT1 and LNC-EPHA6 were strongly increased in patients with DM compared with HC after adjustment for sex and age (p=0.005 and p=0.001, respectively). We also observed increased levels of LNC-RPS24 and LIPCAR. Circulating levels of MALAT1 and LIPCAR were significantly higher in DN patients compared with HC (p=0.008 and p=0.047, respectively) and a trend was observed for LNC-EPHA6 (*Figure 2*). No lncRNAs showed significantly lower levels in DN patients. We also analyzed whether dialysis treatment before transplantation affected circulating lncRNA levels, but no correlation was found (data not shown).

Normalization of lncRNAs in SPKT patients

Given the increased lncRNA levels as a result of DN, we next sought to determine if SPKT would normalize lncRNA levels. Compared with DN patients, levels of MALAT1, LIPCAR, and LNC-EPHA6 were significantly lower in patients with SPKT after adjustment for sex and age (p<0.001, p=0.007, and p=0.037, respectively). LNC-RPS24 did not differ significantly. Although LIPCAR levels did not significantly differ between SPKT and KTx, MALAT1 and LNC-EPHA6 showed higher values in the KTx group compared with the SPKT group, which implies that changes other than kidney function play a role in these altered lncRNA levels.

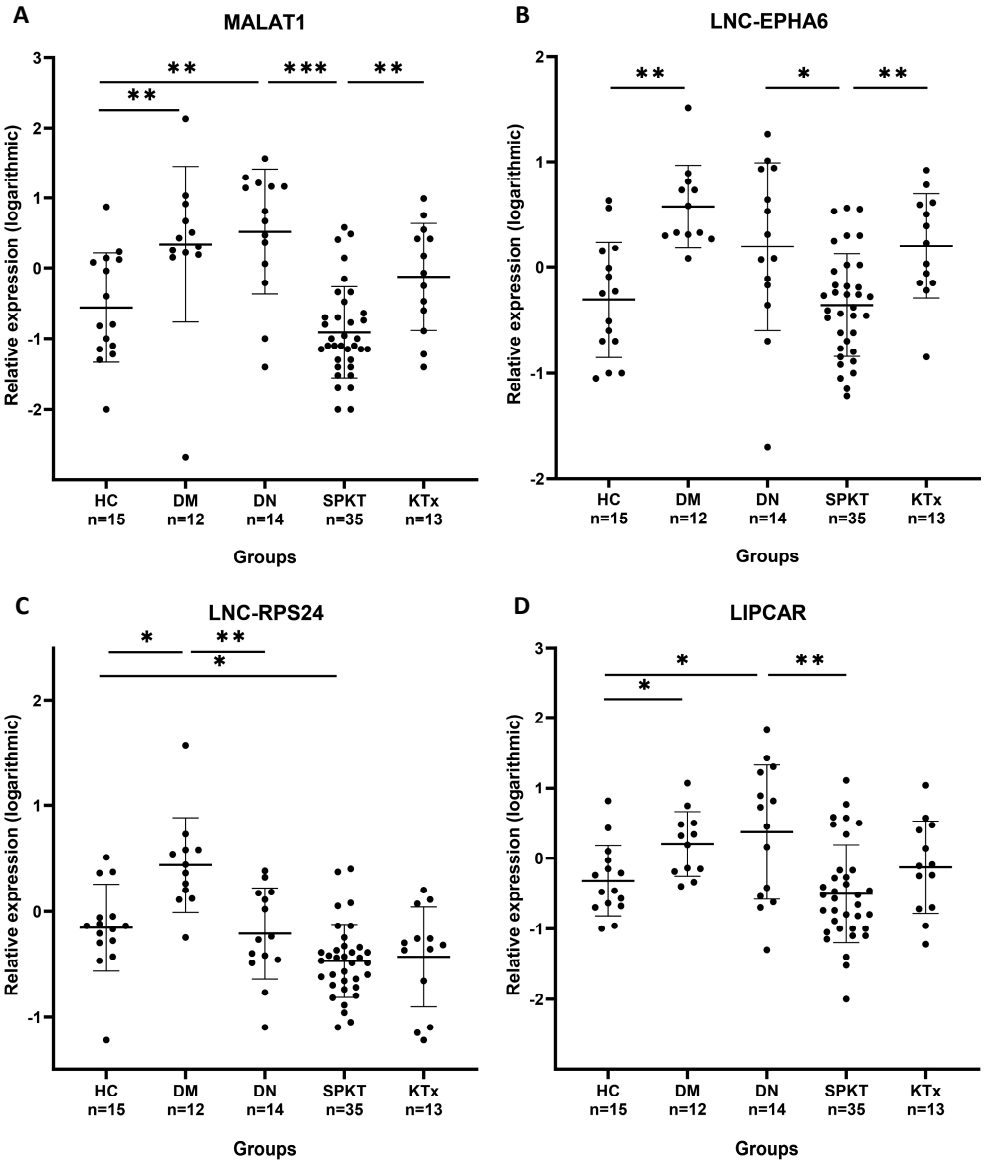

Figure 2. Circulating lncRNA levels are affected by diabetic nephropathy and simultaneous pancreas kidney transplantation. *Relative expression of MALAT1 (A), LNC-EPHA6 (B), LNC-RPS24 (C) and LIPCAR (D) in the cross-sectional cohort; healthy controls (HC; n=15), diabetes mellitus with eGFR > 35ml/min/1.73m2 (DM; n=12), diabetic nephropathy (DN; n=14), simultaneous pancreas-kidney transplantation (SPKT; n=35) and kidney transplantation (KTx; n=13). LncRNA relative expression levels are depicted as logarithmic values. Data are represented as mean ± SD, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.*

Table 2. *Longitudinal study patient characteristics (n=16)*

Characteristics	D0 (n=12)	M1 (n=12)	M6 (n=15)	M12 (n=14)
Sex, *male, n (%)*	9 (75%)	9 (75%)	12 (80%)	12 (86%)
Age *(years)*	44 ± 6	44 ± 6	45 ± 6	45 ± 6
BMI *(kg/m²)*	24.9 ± 3.3	23.9 ± 2.7	24.1 ± 2.0	23.8 ± 2.3
Systolic BP *(mmHg)*	148 ± 19	127 ± 25	135 ± 23	129 ± 15
Diastolic BP *(mmHg)*	86 ± 12	76 ± 14	79 ± 13	78 ± 6
Haemoglobin *(mmol/l)*	7.5 ± 0.7	6.7 ± 1.0	7.3 ± 0.8	7.9 ± 0.9
Haematocrit *(l/l)*	0.36 ± 0.04	0.33 ± 0.05	0.37 ± 0.04	0.39 ± 0.05
Glucose *(mmol/l)*	14.5 ± 6.6	6.4 ± 1.1[1]	5.3 ± 1.4[1]	5.7 ± 1.5[1]
Proteinuria *(g/24 h)* ***median (IQR)***	0.68 (0.36-0.76)	0.74 (0.39-1.40)	0.52 (0.18-0.98)	0.53 (0.14-1.08)

D0 = before transplantation, M1 = 1 month after transplantation, M6 = 6 months after transplantation, M12 = 12 months after transplantation, BMI = body mass index. [1] *p-value <0.05 versus D0.* [2] *p-value <0.05 versus M1.* [3] *p-value <0.05 versus M6.*

Dynamics of lncRNAs after SPKT

To validate the changes of lncRNAs after SPKT, we followed DN patients who received a successful SPKT in time. Plasma samples for detecting lncRNA expression were obtained before (D0) and 1, 6, and 12 months after transplantation (M1, M6, and M12, respectively). The patient characteristics are presented in *Table 2*. Significant improvement of eGFR and significant decline in HbA_{1c} levels after transplantation were observed and are presented in *Figure 3E,F*. MALAT1 and LIPCAR levels decreased during the first year in accordance with the differences between DN and SPKT patients as demonstrated in the cross-sectional study cohort (*Figure 3*). Moreover, they appear to have normalized as early as 1 month after transplantation. LNC-EPHA6 showed the same trend although it was not statistically significant. No further significant changes were observed following the 1-month timepoint, whereas LNC-RPS24 levels did not change within the first year after SPKT.

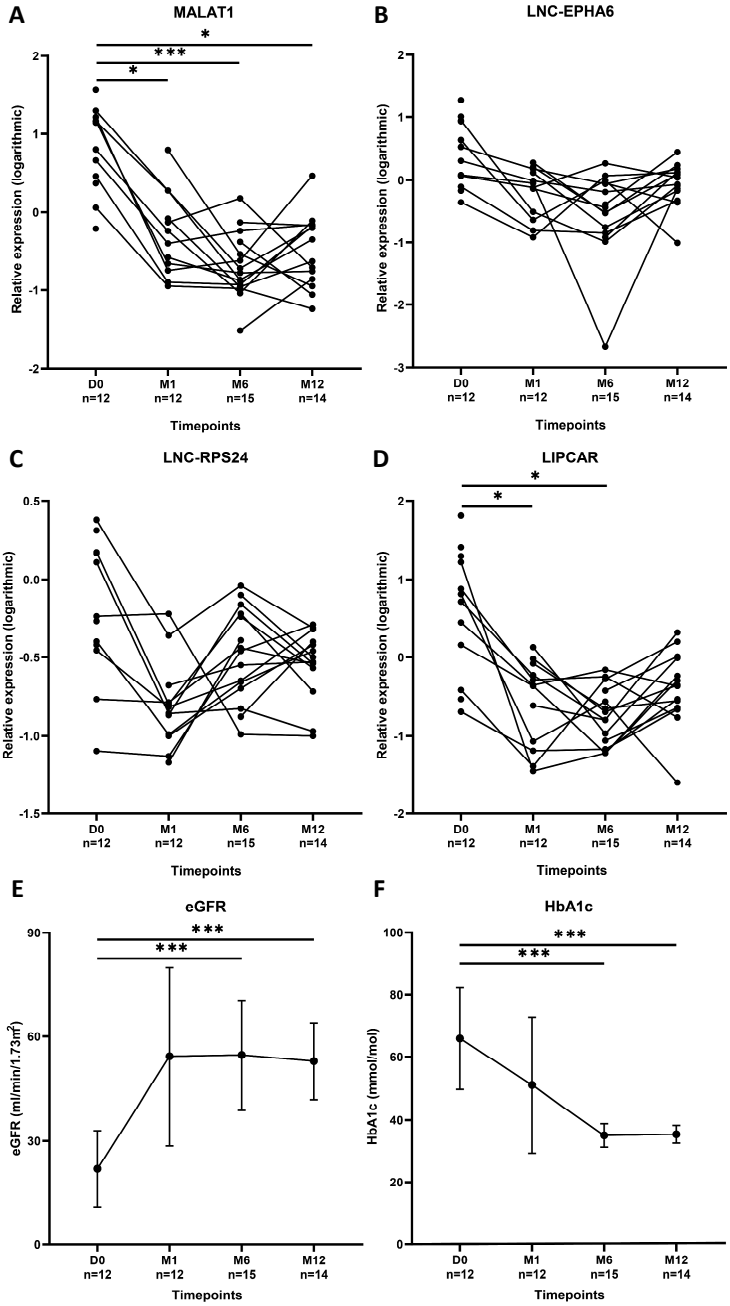

Figure 3. Longitudinal study validates differential lncRNA expression and indicates dynamics. *Relative expression of MALAT1 (A), LNC-EPHA6 (B), LNC-RPS24 (C) and LIPCAR (D) before (D0) and 1, 6 and 12 months (resp. M1, M6 and M12) after simultaneous pancreas-kidney transplantation. LncRNA relative expression levels are depicted as logarithmic values. eGFR (E) improves after transplantation and HbA_{1c} (F) declines to steady levels. Data are represented as mean ± SD, * p-value < 0.05, *** p-value < 0.001.*

LncRNAs associate with soluble thrombomodulin and miRNAs

To assess the relationship between lncRNAs levels and vascular injury, we analyzed their correlation with vascular injury markers sTM and Ang-2. In addition, we assessed the correlation between lncRNAs and previously determined angiogenic miRNA levels in these patients (miR-25, miR-27a, miR-126, miR-130b, miR-132, miR-152, miR-181a, miR-223, miR-320, and miR-326), because previous studies showed that these miRNAs may serve as markers for vascular injury. Moreover, we analyzed the correlation between lncRNAs and kidney function (eGFR) and diabetes (HbA_{1c}). Interestingly, we found that LIPCAR negatively correlated with eGFR while MALAT1 correlated significantly with HbA_{1c}. When we related lncRNA levels to markers of vascular injury, we found sTM to show a positive correlation with LNC-EPHA6 and LIPCAR. Furthermore, miR-27a, miR-130b, miR-152, and miR-340 w ere c orrelated with MALAT1, LNC-EPHA6, and LIPCAR, whereas LNC-EPHA6 also correlated with miR-25, after adjustment for sex, age, and multiple testing (*Table 3*).

Table 3. *Correlation of lncRNAs with kidney function (eGFR), diabetes (HbA_{1c}) and vascular injury markers sTM, Ang-2 and angiogenic miRNAs. Values represent correlation coefficient and p-value.*

	MALAT1	**LNC-EPHA6**	**LIPCAR**	**LNC-RPS24**
eGFR *(ml/min/1.73 m²)*	-0.198 (ns)	-0.244 (ns)	**-0.412 (p=.003)**	-0.132 (ns)
HbA_{1c} *(mmol/mol)*	**0.357 (p=.010)**	0.244 (ns)	0.218 (ns)	0.095 (ns)
Vascular injury markers				
sTM	0.250 (ns)	**0.284 (p=.031)**	**0.342 (p=.009)**	0.091 (ns)
Ang-2	0.069 (ns)	-0.015 (ns)	0.030 (ns)	-0.200 (ns)
Angiogenic miRNAs				
miR-25	-0.062 (ns)	**-0.409 (p=.02)**	0.320 (ns)	-0.301 (ns)
miR-27a	**0.384 (p=.05)**	**0.670 (p<.001)**	**0.616 (p<.001)**	-0.006 (ns)
miR-126	-0.128 (ns)	0.074 (ns)	0.056 (ns)	0.006 (ns)
miR-130b	**0.539 (p<.001)**	**0.711 (p<.001)**	**0.658 (p<.001)**	0.026 (ns)
miR-132	0.243 (ns)	0.361 (ns)	0.285 (ns)	0.221 (ns)
miR-152	**0.447 (p=.004)**	**0.557 (p<.001)**	**0.503 (p<.001)**	-0.024 (ns)
miR-181a	0.074 (ns)	0.247 (ns)	0.216 (ns)	0.052 (ns)
miR-223	-0.297 (ns)	0.004 (ns)	-0.095 (ns)	0.002 (ns)
miR-320	0.252 (ns)	0.232 (ns)	0.269 (ns)	0.118 (ns)
miR-326	0.082 (ns)	0.245 (ns)	0.261 (ns)	0.084 (ns)
miR-340	**0.532 (p<.001)**	**0.657 (p<.001)**	**0.603 (p<.001)**	-0.011 (ns)
miR-574	-0.332 (ns)	-0.240 (ns)	-0.273 (ns)	0.070 (ns)
miR-660	0.319 (ns)	0.109 (ns)	0.119 (ns)	0.169 (ns)

sTM=soluble thrombomodulin, Ang-2=Angiopoietin-2.

Discussion

This study shows that plasma levels of specific lncRNAs (MALAT1 and LIPCAR) are significantly higher in patients with DN compared with in healthy individuals. Both MALAT1 and LIPCAR, as well as LNC-EPHA6, are significantly lower in patients who received an SPKT compared with DN patients. This phenomenon is confirmed in our longitudinal study where these lncRNAs show a significant decrease during the first year after transplantation. In addition, MALAT1, LNC-EPHA6, and LIPCAR strongly associate with sTM and angiogenic miRNAs, suggesting that the identified lncRNAs are associated with vascular injury.

Interestingly, MALAT1 and LNC-EPHA6 levels decreased after SPKT but exhibited higher levels in patients who received a KTx only. This suggests that the reduction in plasma levels of these lncRNAs is not related to restoration of renal function but might be associated with glycemic control. In line with this, we did find a significant correlation between MALAT1 and HbA_{1c} levels. The clear difference in MALAT1 levels between the HC and DM group further supports this finding. In contrast, LIPCAR levels did not statistically differ between the SPKT and KTx groups, suggesting that LIPCAR levels are more dependent on renal function. This is confirmed by the strong correlation of LIPCAR with eGFR, whereas MALAT1, LNC-EPHA6, and LNC-RPS24 do not correlate with kidney function. Nonetheless, although we have a well-defined cohort, further studies are necessary to validate these findings, as group sizes in the current study are limited. However, the longitudinally study also serves as an internal validation to confirm the results of the cross-sectional study while it illustrates the natural course of lncRNAs after restoring endogenous insulin secretion and kidney function.

Furthermore, we cannot exclude that altered levels of lncRNAs were caused by the immunosuppressive therapy that patients with SPKT received, although the KTx group serves as a control group with comparable immunosuppressive therapy. Because MALAT1 and LNC-EPHA6 levels differ significantly in SPKT compared with KTx, this suggests that these altered lncRNAs levels are not due to the immunosuppressive therapy.

In our study, we selected 22 candidate lncRNAs, of which only 9 were detectable using RT-qPCR. These included MALAT1, which was below the detection threshold in our microarray pilot. LIPCAR did show increased plasma levels in patients with DN compared with healthy controls in the pilot study, but this was not statistically significant due to a large range in data values (while the group sizes in the pilot were limited to n=6). Of the 9 lncRNAs that were detectable using RT-qPCR, only 4 of these were detectable in the majority of the samples (>95%) and therefore further analyzed to enable a robust interpretation of these lncRNAs. Nonetheless, probably due to the fact that lncRNAs are often expressed at very low levels,[40] the majority of lncRNAs were either undetectable or sporadically detectable, which is consistent with previous reports,[41] suggesting only highly abundant lncRNAs may prove to be useful as biomarkers.

Given that DN and SPKT strongly associate with vascular injury,[1] we analyzed the relation of lncRNA levels with previously assessed markers of vascular injury (sTM, Ang-2, and angiogenic miRNAs).[7] We observed a correlation of LIPCAR and LNC-EPHA6 with sTM, whereas we found several strong correlations of LIPCAR, MALAT1, and LNC-EPHA6 with a specific subset of angiogenic miRNAs (miR- 27a, miR-130b, miR-152, and miR-340). Although these associations may prove to be not causally related, it is interesting that, for example, MALAT1 has been described previously in the pathogenesis of several vascular diabetic complications, such as DR and cardiomyopathy.[42-44] The reduced MALAT1 levels after SPKT suggest an improved state of vascular health that may associate with diminished development of these secondary diabetic complications. Moreover, LIPCAR was previously described to be correlated with the presence of heart failure and predicts subsequent patient survival,[30] whereas in DM patients, LIPCAR is strongly correlated with left ventricular diastolic dysfunction, waist circumference, and plasma fasting insulin.[45] Although LNC-EPHA6 has not been described previously, lncRNAs are often coexpressed and coregulated with their neighboring genes.[46] As such, it may be speculated that LNC-EPHA6 function relates to the biological role of EPHA6, which is part of a family of EPH receptor tyrosine kinases, which interact with ephrins and hereby regulate important processes such as angiogenesis.[47,48] In addition, lncRNAs have often been described to influence miRNA levels by serving as an miRNA sponge,[49] providing a potential link between angiogenic miRNAs and differentially regulated lncRNAs in the context of DN. Furthermore, circulating lncRNAs are carried in extracellular vesicles (EVs) and as such may contribute to vascular injury via distant cell-cell communication. In fact, EV-containing lncRNAs and miRNAs are described as important factors in communication between organs in diabetes.[50] It is important to note that we previously described that the RNA obtained using our isolation methods contains all EV RNA, as evidenced by, among others, CD63 expression, which is a marker of EVs and electron microscopy confirmation of EV content of plasma.[51] Interestingly, we see a strong correlation of lncRNAs MALAT1, LNC-EPHA6, and LIPCAR with miRNA-27a, miRNA-130b, miRNA-152, and miRNA-340, which may be explained by the coappearance of these noncoding RNAs in the same EV. Indeed, these miRNAs have been previously demonstrated to be involved in cell-cell communication via EV.[51-54] Taken together, these results emphasize the potential of lncRNAs in the pathogenesis of the disease, whereas both MALAT1 and LIPCAR, as well as LNC-EPHA6, may play an important role in angiogenesis and the development of vascular injury, suggesting changed circulating levels of these lncRNAs may reflect vascular injury in the context of DN and SPKT.

In conclusion, we are the first to demonstrate that several lncRNAs are altered in DN patients and normalize after SPKT. Our data suggest that certain lncRNAs reflect (micro)vascular damage and that these lncRNAs might provide better insight in the pathophysiology of DN and SPKT and could potentially serve as a novel tool to monitor vascular integrity.

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Supplementary information

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Chapter 4

Circulating long noncoding RNA LNC-EPHA6 associates with acute rejection after kidney transplantation

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Abstract

Background

Acute rejection (AR) of a kidney graft in renal transplant recipients is associated with microvascular injury in graft dysfunction and, ultimately, graft failure. Circulating long noncoding RNAs (lncRNAs) may be suitable markers for vascular injury in the context of AR.

Methods

Here, we first investigated the effect of AR after kidney transplantation on local vascular integrity and demonstrated that the capillary density markedly decreased in AR kidney biopsies compared to pre-transplant biopsies. Subsequently, we assessed the circulating levels of four lncRNAs (LNC-RPS24, LNC-EPHA6, MALAT1, and LIPCAR), that were previously demonstrated to associate with vascular injury in a cohort of kidney recipients with a stable kidney transplant function (n=32) and recipients with AR (n=15). The latter were followed longitudinally six and 12 months after rejection.

Results

We found higher levels of circulating LNC-EPHA6 during rejection, compared with renal recipients with a stable kidney function (p=0.017), that normalized one year after AR. In addition, LNC-RPS24, LNC-EPHA6, and LIPCAR levels correlated significantly with the vascular injury marker soluble thrombomodulin.

Conclusions

We conclude that AR and microvascular injury are associated with higher levels of circulating LNC-EPHA6, which emphasizes the potential role of lncRNAs as biomarker in the context of AR.

Introduction

Acute rejection (AR) is considered to be a prominent cause of graft failure in the first year after transplantation in kidney transplant recipients,[1,2] although the long-term consequences of AR remain a subject of discussion. Despite better screening and improved immune suppressive therapies, rejection is still suspected to cause a significant proportion of death censored graft failure after kidney transplantation.[3,4] Previous research showed a prolonged effect on kidney function deterioration as well as graft survival after a rejection episode.[2] Microvascular endothelial cells ECs) are very susceptible to injury, that can result from episodes of AR. Following the alloimmune response, cytokines and growth factors are produced that can lead to EC activation and microvascular destabilization.[5-10] These rejection-associated events can result in perpetual EC damage and promotion of (aberrant) angiogenesis within the allograft.[5,7,9] Together, these insults can lead to the loss of the microvasculature, chronic ischemia and cell death,[11,12] and ultimately, to the development of interstitial fibrosis/tubular atrophy and graft dysfunction.[5,6,9] Therefore, monitoring the course of microvascular injury after rejection could be beneficial in deciding on the best treatment strategies. Previously, we found the vascular injury markers soluble thrombomodulin (sTM) and Angiopoietin-2 (Ang-2) to increase upon AR. sTM normalized in the first year after AR, while Ang-2 remained elevated.[13] Noncoding RNA, such as micro RNAs (miRNA) and long noncoding RNAs (lncRNA)are increasingly recognized to play an important role in vascular injury.[14] The functions of lncRNAs appear to be very diverse as they can bind DNA, proteins, and other RNAs. E.g. lncRNAs have been demonstrated to serve as a scaffold for transcription factors or can assist chromatin-modifying enzymes, thereby regulating gene expression.[15] LncRNAs were also found to be important for miRNA processing, (alternative) splicing, translation and post-transcriptional regulation, for instance via sponging miRNAs.[16,17] In addition, lncRNAs can be promising biomarkers in a variety of vascular diseases and kidney injury.[14,16] Furthermore, lncRNAs have previously been associated with AR,[18] but their dynamics after rejection have not been studied before. Earlier, we described that specific lncRNAs (MALAT1, LNC-RPS24, LNC-EPHA6, and LIPCAR) associate with microvascular damage and angiogenic factors in patients with diabetic nephropathy that received simultaneous kidney-pancreas transplantation,[19] but their relation with AR and associated vascular damage is unclear. As such, in this study we first explored the relation of AR with local microvascular injury. Then, in a cross-sectional study of patients with T cell mediated AR, we analyzed selected vascular injury related lncRNAs as potential biomarkers for vascular damage in the context of kidney transplant rejection and assess the dynamics in these lncRNAs after rejection.

Materials and methods

Renal Biopsy Study

Renal biopsies were selected from patients that had a biopsy proven acute renal allograft rejection, as previously described.[20] Patient and transplantation characteristics are summarized in *Supplementary Table S1*. Frozen biopsy tissue sections (4 µm) were fixed in acetone, endogenous peroxidase was blocked with H2O2, and slides were blocked with 1% bovine serum albumin and 5% normal human serum in PBS. Sections were then incubated with specific antibodies directed against CD34 (BD Biosciences, Breda, The Netherlands) and CD73 (BD Biosciences, Breda, The Netherlands) followed by appropriate secondary antibodies that were HRP-conjugated (Jackson Immunoresearch, Westgrove, PA, USA). Stainings were visualized using Nova RED (Vector Labs, Peterborough, UK). Quantification of immunohistological staining results was performed using image J software.

Patient study cohort

A total of 47 patients were enrolled in a cross-sectional, observational, single center study. All patients were transplanted between 2006 and 2012 in the Leiden University Medical Center (LUMC) in Leiden, The Netherlands. The cohort consisted of 2 groups, namely renal transplant recipients with AR (n=15) and a control group consisting of renal recipients 12 months after transplantation without rejection and with a stable kidney transplant function (n=32). In addition, recipients from the rejection group were followed longitudinally. Plasma samples were obtained at 6 and 12 months after rejection. The cohort has been described earlier where analysis of circulating Ang-2 and sTM in plasma was performed.[13] All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of The Leiden University Medical Center (P09.141).

Immunosuppressive drugs, rejection and rejection treatment

All patients received immunosuppressive drug therapy according to the standard of care at the time of transplantation. IL-2 receptor inhibitor as induction therapy was the standard of care and alemtuzumab was administered in case the treating physician expected a higher risk of rejection. The presence and type of rejection was assessed using the Banff classification. The choice for a specific rejection treatment was made according to the standard of care at the time of rejection.[13]

RNA isolation

The RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) was used with an adapted protocol, to isolate total RNA from 200 µL plasma. In summary, using 800 trizol µL reagent (Invitrogen, Breda, The Netherlands), the plasma/Trizol sample was centrifuged for 15 min (15,000 g) after the addition of 160 µL chloroform. Then, 100% ethanol (1.5 volume) was added to the

aqueous phase and transferred to a MinElute Spin column (Qiagen) followed by centrifugation for 15 s (18,000 g). Subsequently, 700 µL RWT bu_er and twice 500 µL RPE bu_er was used to wash the column. The column was centrifuged (18,000 g) for 15 s after the first two washing steps and 2 min (18,000 g) after the third washing step. 15 µL RNase-free water was added for elution of the RNA.

RT-qPCR

To quantify circulating lncRNA levels we performed RT-qPCR. Isolated RNA was reverse transcribed using Iscript (Biorad) according to the protocol of the manufacturer. RT-qPCR of target genes was done using SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA). The primer sequences of target lncRNAs are given in *Supplementary Table S2*.

Statistical analyses

Categorical data are described as total count and percentages, parametric data as mean ± standard deviation (SD), and non-parametric data as median and interquartile range (IQR). Testing for differences of baseline characteristics was performed by using Fishers exact test for categorical data and the unpaired t test and Mann-Whitney U test for parametric and non-parametric data. Circulating lncRNA levels were normalized by the double delta CT method to miR-16 and subsequently transformed logarithmically (with base 10). The logarithmic relative expression of all three lncRNAs was normally distributed. In the longitudinal study the data was analyzed by using a linear mixed model analysis. Analysis of correlations between the lncRNAs and vascular markers was performed using Spearman rank correlation. A p-value <0.05 was considered to be statistically significant. SPSS version 23.0 (SPSS, Inc., Chicago, IL, USA) was used for the data analysis and Graphpad Prism version 8.0 (Graphpad Prism Software, Inc., San Diego, CA, USA).

Results

Decreased capillary density in acute rejection biopsies

To assess the impact of AR on the local capillary density in the kidney, we quantified the number of endothelial cells (EC) and pericytes in archival acute rejection biopsies by immunohistochemical staining of the EC for CD34 antigen and the pericytes for the CD73 marker (resp. n=102 and n=29). Subsequently, we compared these parameters to the available pre-transplant biopsies (resp. n=78 and n=66) of these patients.[20] Patient characteristics can be found in *Supplementary Table S1*. As shown in *Figure 1*, we observed a strong decrease in both the number of endothelial cells (~2.5-fold, p<0.001) as well as pericytes (~6-fold, p<0.001) in AR, indicating loss of the peritubular capillary network in AR.

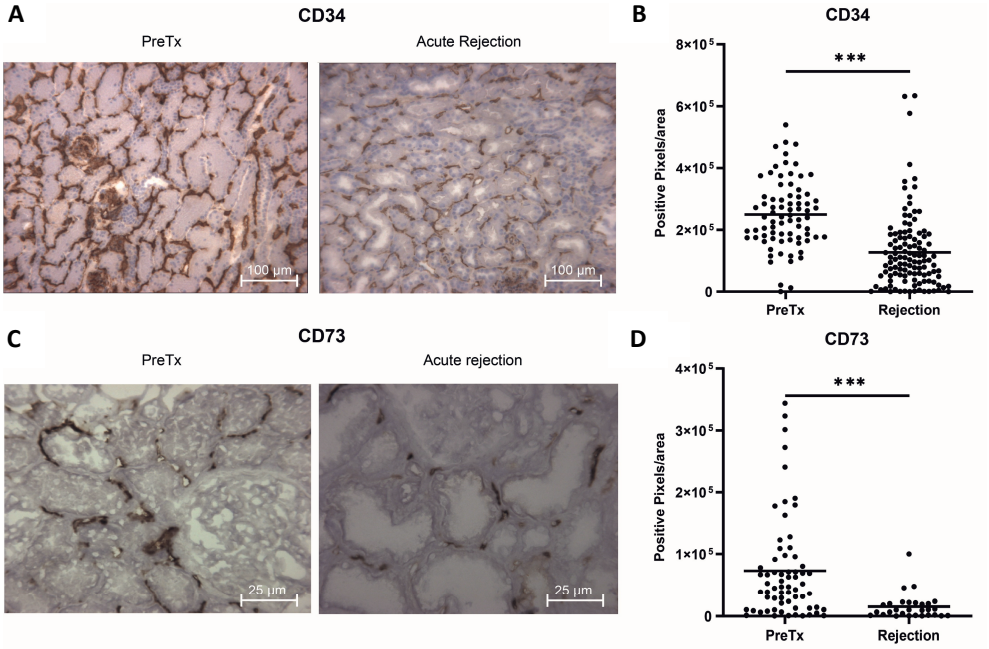

Figure 1. Decreased capillary density after acute rejection. *(A) Representative images of CD34 staining for pre-transplantation and acute rejection (AR) biopsies. (B) Quantification of CD34 staining (PreTx n=78; AR, n=102). (C) Representative images of CD73 staining for pre-transplantation and acute rejection biopsies. (D) Quantification of CD73 staining (PreTx, n=66; AR, n=29).*

Patient characteristics of cross sectional and longitudinal AR study population

Next, we sought to investigate the relation of circulating lncRNAs with AR. To that end, we included plasma samples of a different cross-sectional study cohort that included patients with acute T cell mediated rejection and a control group of patients with stable kidney transplant function after transplantation (hereafter mentioned as 'stable'). In addition, AR patients were studied longitudinally at 6 and 12 months after rejection to determine the dynamics after AR. The baseline characteristics of the transplant recipients in this cohort are described in *Table 1*. Most common causes of initial kidney failure before transplantation were autosomal dominant polycystic kidney disease (23%), focal segmental glomerulosclerosis (17%) and IgA nephropathy (13%). The mean time after transplantation (12 months) was comparable. Immunosuppressive regimen did not differ significantly. eGFR was lower and proteinuria higher in patients with AR, compared with stable patients (resp. $p<0.001$ and $p=0.003$). Factors that can influence the amount of vascular injury next to rejection, such as donor age, dialysis before transplantation, and months since transplantation, did not differ significantly. Incidence of active smokers was 7% in AR patients and 13% in stable patients. Panel reactive antibodies (PRA), mismatch, immunosuppressive regimen and the presence of previous transplantations did not differ between stable patients and patients with AR.

Table 1. *Cross-sectional study patient characteristics of patients with a stable kidney transplant function (stable) and patients with acute rejection (AR).*

Characteristics	**Stable (n=32)**	**AR (n=15)**	**p-value**
Sex, *male, n (%)*	21 (66%)	10 (67%)	1.00[1]
Age, *years ± SD*	51 ± 14	54 ± 12	0.35[2]
BMI *(kg/m²)*	26.4 ± 4.6	24.4 ± 3.5	0.15[1]
Preemptive, *n (%)*	16 (50%)	5 (33%)	0.36[1]
Months since KTx, *median (IQR)*	12 ± 1	12 ± 15	0.97[2]
Panel reactive antibody >5%, *n (%)*	6 (19%)	1 (7%)	0.40[1]
Previous transplantations, *n (%)*	2 (6%)	3 (20%)	0.31[1]
Mismatch A / B / DR, *mean*	1.0 / 1.2 / 0.8	0.9 / 1.3 / 1.0	0.76 / 0.81 / 0.63[1]
Donor characteristics			
Sex, *male, n (%)*	11 (34%)	7 (47%)	0.52[1]
Age, *years ± SD*	50 ± 17	47 ± 12	0.64[2]
Induction therapy, *n (%)*			0.54[1]
Alemtuzumab	3 (9%)	0	
IL-2 receptor inhibitor	29 (91%)	15 (100%)	
Immunosuppressive drugs, *n (%)*			
Tacrolimus	22 (69%)	8 (53%)	0.20[1]
Cyclosporine	5 (16%)	3 (20%)	1.00[1]
Prednisone	32 (100%)	14 (93%)	0.32[1]
Mycophenolate mofetil	25 (78%)	8 (53%)	0.07[1]
Everolimus	6 (19%)	1 (7%)	0.40[1]
Acute rejection therapy, *n (%)*			
ATG	-	2 (13%)	
methylprednisolone	-	10 (67%)	
methylprednisolone + ATG	-	2 (13%)	
methylprednisolone + alemtuzumab	-	1 (7%)	
eGFR (ml/min/1.73m²)	54 ± 12	34 ± 14	<0.001[2]
Proteinuria (g/24h), median *(IQR)*	0.17 (0.13-0.25)	0.36 (0.23-1.19)	0.003[3]

[1] Fisher's exact test, [2] unpaired t-test, [3] Mann-Whitney U test, KTx = kidney transplantation.

Patients with AR had interstitial rejection, with or without involvement of the vasculature, and were treated with methylprednisolone (67%), ATG alone (13%), or a combination of methylprednisolone and ATG (13%) or alemtuzumab (13%).

Circulating LNC-EPHA6 levels directly correlate with acute rejection

In order to assess the relationship between AR and vascular injury related lncRNAs LNC-RPS24, MALAT1, LNC-EPHA6, and LIPCAR, circulating levels of these lncRNAs were measured in stable patients and AR patients. In this cohort, MALAT1 levels were only detectable in less than 30% of patients and therefore not included in further analyses.

Relative expression of circulating LNC-EPHA6 was significantly higher in patients with AR, compared with stable patients (p=0.017; *Figure 2*). LNC-RPS24 and LIPCAR showed a similar trend, although these differences did not reach statistical significance (resp. p=0.11 and p=0.16).

Circulating LNC-EPHA6 decreases in the first year after acute rejection

Since vascular damage persists after a rejection episode, patients with AR were followed longitudinally to study the dynamics of lncRNAs in the first year after AR. Elevated levels of circulating LNC-EPHA6 persisted until six months after AR (p<0.001) and decreased significantly one year after rejection, although LNC-EPHA6 levels at one year after rejection remained slightly higher levels than in stable patients (p=0.03; *Figure 2*). LIPCAR showed a similar pattern without reaching significance (p=0.16), while LNC-RPS24 increased one year after transplantation (*Figure 2*). eGFR did not change significantly the year after AR.

LNC-RPS24, LNC-EPHA6 and LIPCAR correlate with soluble thrombomodulin

In order to analyze the association of lncRNAs with vascular injury due to AR, we studied the correlation of LNC-RPS24, LNC-EPHA6, and LIPCAR with vascular injury markers sTM and Ang-2 that were previously assessed.[13] There, we showed a significant increase of sTM levels in patients with acute rejection, followed by a subsequent normalization one year after transplantation, while the ratio between Ang-2 and Ang-1 (mainly determined by Ang-2) significantly increased during AR without significant changes afterwards. Here, no significant associations were found between LNC-RPS24, LNC-EPHA6, and LIPCAR with Ang-2. However, interestingly, LNC-RPS24, LNC-EPHA6, and LIPCAR correlated positively with sTM (*Table 2*).

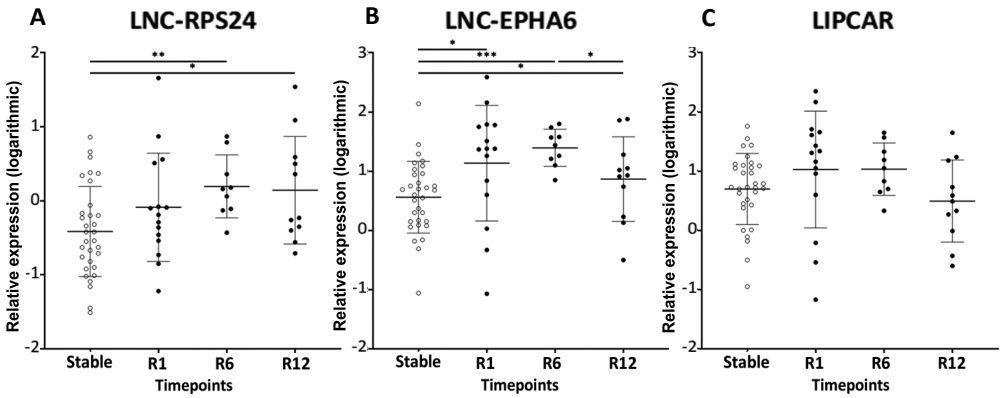

Figure 2. Circulating lncRNA levels are effected by acute rejection. *Relative expression of LNC-RPS24 (A), LNC-EPHA6 (B), and LIPCAR (C) in the cross-sectional cohort; kidney recipients with a stable kidney function (Stable; n=32), kidney recipients with acute rejection at the time of rejection (R0; n=15), and 6 and 12 months after rejection (R6 and R12; n=9 and n=11). Data are presented as mean ± SD, * p-value <0.05, ** p-value <0.01, *** p-value <0.001.*

Table 2. *Correlation of lncRNAs with vascular injury markers sTM, Ang-2. Values represent correlation coefficient and p-value.*

	LNC-RPS24	LNC-EPHA6	LIPCAR
Vascular injury markers			
sTM	0.331 (p=0.035)	0.383 (p=0.013)	0.321 (p=0.041)
Ang-2	ns	ns	ns

sTM = soluble thrombomodulin, Ang-2 = angiopoietin-2.

Discussion

Our study shows that levels of circulating LNC-EPHA6 are significantly higher in patients with T cell-mediated AR after renal transplantation, compared with kidney transplant recipients with a stable allograft function. LNC-EPHA6 remains elevated after AR, followed by a decrease one year after rejection. LIPCAR shows a similar pattern, but did not reach statistical significance. In addition, LNC-EPHA6, LIPCAR, and LNC-RPS24 correlate with the vascular injury marker sTM. This suggests that, in particular, LNC-EPHA6 may be related to microvascular damage, of which we confirmed its relation to AR by demonstrating a significantly lower presence of endothelial cells and pericytes in our renal biopsy study.

LNC-EPHA6 was earlier found to relate to diabetic nephropathy,[19] but was not studied in the context of AR before. Our finding of higher LNC-EPHA6 levels in patients with AR compared with patients without AR provided proof of principle of the biomarker potential of lncRNAs in AR. However, here we analyzed four pre-selected lncRNAs, thus analyses of other lncRNAs in AR may yield additional associations and may potentially be important for prediction of (vascular injury after) AR. This is in line with two other studies that showed an association between lncRNAs and AR that suggested their value for diagnosis of AR in kidney transplantation.[21,22] Moreover, in a rat study, the lncRNA PRINS was shown to be significantly up-regulated in kidneys of rats with cold ischemia-elicited allograft rejection, compared with rats without rejection.[23] In addition, lncRNAs may also be of value in predicting the development of chronic damage after kidney transplantation.[24]

Interestingly, levels of circulating LNC-EPHA6 decrease after AR, while eGFR remains stable. This substantiates that changes in LNC-EPHA6 are likely not to be related to changes in kidney function, but other factors in the pathogenesis of AR, such as persisting microvascular injury. This suggestion is supported by the strong correlation with sTM. However, although significant differences between immunosuppressive drug regimen were not observed, we cannot exclude that differences in rejection treatment altered levels of circulating lncRNAs. Furthermore, an association with the function of the EPHA6 gene might be possible, since lncRNAs are frequently co-regulated and co-expressed with their

neighboring genes.[25] The EPHA6 gene is part of a EPH receptor tyrosine kinases family, and thereby interacts with ephrins which subsequently regulates several cellular processes including angiogenesis.[26,27]

LIPCAR showed a similar trend after rejection as LNC-EPHA6. This could suggest a similar association as LNC-EPHA6 with rejection. However, changes in LIPCAR did not reach statistical significance due to a large variation. Analysis of LIPCAR in a larger cohort of patients with AR may confirm the link with vascular injury in rejection, since the size of our groups limits the interpretation of LIPCAR in our study. Circulating LNC-RPS24 was only marginally higher in rejection, but increased six months after year after rejection and remained higher. Although speculative, this may be the result of persistent vascular injury after AR or a consequence of the rejection treatment. Lastly, we found LncRNA MALAT1 to be only detectable in less than 30% of the patients in our cohort. Previously, MALAT1 was however detectable in most diabetes mellitus patients,[19] suggesting that diabetes mellitus may increase circulating Malat1 levels. However, next to the previously mentioned limited group size, a relatively large spread of lncRNA levels within groups limits the possibility of drawing robust conclusions. The interpretation of subtle changes (e.g. correlation of lncRNAs with the specific Banff classification score for tubulitis, interstitial inflammation, and intimal arteritis) is difficult and larger groups are necessary for the identification of a specific lncRNA as a novel biomarker. However, differences in lncRNAs levels point out the interesting possible added value of lncRNAs in the context of acute cellular rejection. Identification of lncRNAs in the context of antibody-mediated rejection would be interesting as well, since this rare condition also has major implications for the amount of vascular injury.

In conclusion, LNC-EPHA6 is higher in kidney transplant recipients with rejection, compared with those without. This is the first study that shows changes in vascular injury related lncRNAs the first year after rejection. The results suggest that lncRNAs may reflect (micro)vascular damage in the context of rejection and emphasizes the potential role of lncRNAs as biomarkers to monitor vascular injury in kidney transplant rejection.

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Supplementary information

Supplementary table S1. *Patient and transplantation characteristics of patients in the renal biopsy study (n=102)*

Variable	Mean ± SD; count (%)
Recipient	
Age	47.0 ± 12.6
Age =< 50 years	59 (57.8)
Age > 50 years	43 (42%)
Sex (male)	68 (67%)
Sex (female)	34 (33%)
donor	
Age	46.2 ± 13.8
Age =< 50 years	58 (57%)
Age > 50 years	44 (43%)
Sex (male)	39 (38%)
Sex (female)	63 (62%)
Transplantation type	
Living	25 (25%)
Post mortal	77 (75%)
Re-transplantation	15 (18%)
Induction therapy	31 (30%)
PRA	
0-5%	34 (33%)
>5%	68 (67%)
Mismatches Class I	
0-2	78 (80%)
>2	20 (20%)
Mismatches Class II	
0	32 (33%)
1-2	65 (67%)
DGF (need for dialysis)	29 (28%)
Need for antibody therapy*	49 (48%)

*PRA = panel reactive antibodies, DGF = delayed graft function. * 48% of patients with rejection required antibody therapy (antithymocyte globulin) due to a second rejection episode or insufficient response to steroid treatment.*

Supplementary table S2. *Used primer sequences of lncRNAs.*

Primer lncRNA	Sequence
hsa-malat1-fw (MALAT1)	ACCATGGCACTTTCTCCTG
hsa-malat1-rev (MALAT1)	CCCATCACTGAAGCCCACAG
hsa-G003293-fw (LNC-RPS24)	GACGTCGCTATGAACGCTTG
hsa-G003293-rev (LNC-RPS24)	CCAGGTGGGGAGTTTGACTG
hsa-lnc-EPHA6-1:1-fw (LNC-EPHA6)	ATGTTATGCCCGCCTCTTCA
hsa-lnc-EPHA6-1:1-rev (LNC-EPHA6)	TCAGTATTAGAGGCACCGCC
hsa-lipcar-fw (LIPCAR)	TAAAGGATGCGTAGGGATGG
hsa-lipcar-rv (LIPCAR)	TTCATGATCACGCCCTCATA

Chapter 5

Single antigen testing to reduce early antibody-mediated rejection risk in female recipients of a spousal donor kidney

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Abstract

Background

Female recipients of a spousal donor kidney transplant are at greater risk of donor-specific pre-immunization, which may increase the risk of acute antibody-mediated rejection (ABMR).

Methods

We assessed the incidence of early ABMR (within two weeks after transplantation), risk factors for ABMR and graft function in 352 complement-dependent cytotoxicity test-negative LURD transplant recipients, transplanted between 1997-2014 at the Leiden University Medical Center in The Netherlands. Risk factors for immunization were retrieved from the health records. As methods to screen for preformed donor-specific antibodies (pDSA) have developed through time, we retrospectively screened those with ABMR for pDSA using pooled-antigen bead (PAB) and single-antigen bead (SAB) assays.

Results

The cumulative incidence of rejection in the first six months after transplantation was 18% (TCMR 15%; early ABMR 3%). Early ABMR resulted in inferior graft survival and was more common in women who received a kidney from their spouse (10%) than in other women (2%) and men (<1%). The SAB assay retrospectively identified pDSA in seven of nine cases of early ABMR (78%), while the PAB detected pDSA in only three cases (33%).

Conclusions

Seeing that early ABMR occurred in 10% of women who received a kidney from their spouse, a SAB assay should be included in the pre-transplant assessment of this group of women, regardless of the result of the PAB assay.

Introduction

Kidney transplantation improves life expectancy and quality of life for patients with end-stage kidney disease, as compared with dialysis.[1-3] There is an ongoing shortage of deceased donor kidneys that are suitable for transplantation. This has contributed to an increase in living unrelated kidney donation (LURD).[4-6] In the Netherlands up to 60% of the annual transplants now stem from a living kidney donor. A sizeable proportion receives a kidney from a spouse.[7] This is encouraged by several studies that have documented excellent outcomes.[8,9] However, early antibody-mediated rejection (ABMR) can adversely affect outcome.[10-14] ABMR is a result of the formation of antibodies, directed against human leukocyte antigen (HLA) or non-HLA antigens of the donor. Development of ABMR during the first weeks after transplantation suggests the presence of preformed donor specific antibodies (pDSA) and/or dormant HLA specific B cell memory. It is known that women may have more pDSA from previous pregnancies[15] and that the presence of pDSA in the absence of a positive complement-dependent cytotoxicity (CDC) test results in a higher risk of acute rejection and subsequent graft loss.[16] On the other hand, not all low titer pDSA are harmful, but nevertheless may prohibit transplantation. There is no effective therapy to treat ABMR. Plasma exchange, and/or intravenous immune globulin, and glucocorticoids are considered as standard of care, though evidence for these treatment options is scarce and mainly based on small studies and expert consensus.[17]

It is paramount to optimize the pre-transplant assessment of the risk for acute ABMR in LURD. Therefore, the aim of this study was to assess the incidence of early ABMR in LURD and to identify risk factors for ABMR, in particular relevance of pDSA. Furthermore, we studied the effect of early ABMR on subsequent graft function and kidney graft loss (GL).

Materials and methods

Study design and population

This single center, observational, cohort study consisted of all LURD recipients of a blood type (ABO) compatible renal allograft at the Leiden University Medical Center (LUMC) transplanted between 1997 and 2014. The cohort consisted of 352 recipients, including 35 repeat transplants (10%). The majority (85%) had been transplanted after 2004 (*Supplementary Figure 1*). Based on the recipient-donor relationship, the population was divided into four groups: female recipients with either a spousal male donor (n=61), a non-spousal male donor (n=36) or a female donor (n=46) and male recipients (n=209) (*Figure 1*).

Clinical data was obtained from the departmental database containing information that is updated regularly and sent to the Dutch Organ Transplant Registry. This type of retrospective study with data from a registry was exempt from approval from an ethics board. The study was performed in accordance with the FEDERA Code of Conduct.[18]

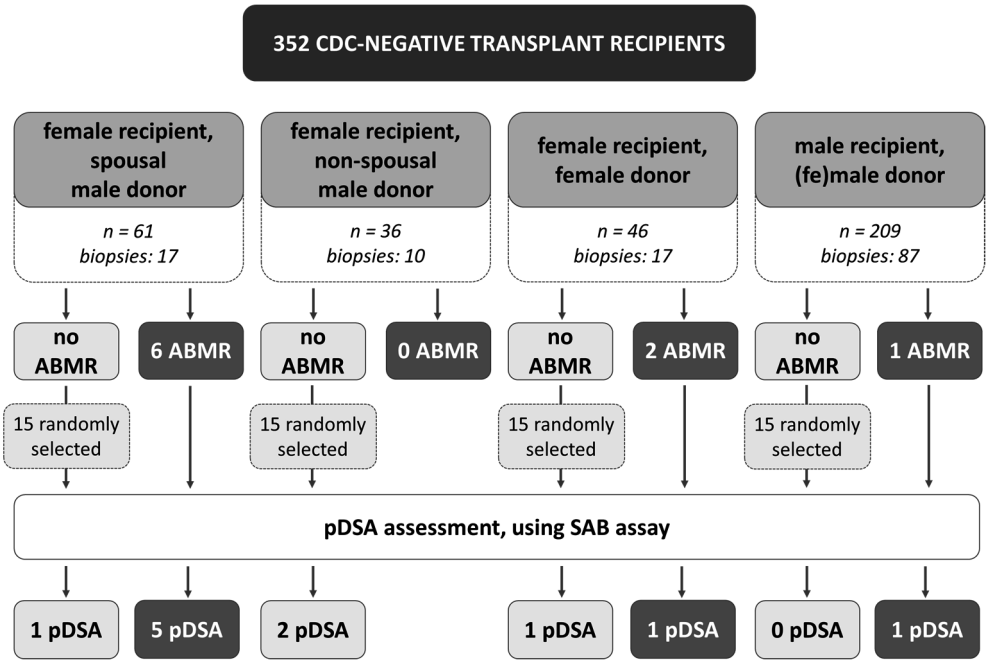

Figure 1. Schematic representation of the cohort and presence of preformed donor specific antibodies (pDSA). *The cohort, consisting of 352 renal recipients with a negative complement-dependent cytotoxicity (CDC) test, was divided into four groups; female recipient with either a spousal male donor (n=61), a non-spousal male donor (n=36) or a female donor (n=46), and male recipients (n=209). All patients with early antibody-mediated rejection (ABMR), supplemented with a randomly selected group (n=60) of patients without ABMR, were tested with a single antigen bead (SAB) assay. The randomly selected group consisted of 56 patients from the 'no rejection' group and 4 from the 'TCMR' group. All four patients with pDSA in the randomly selected group were patients from the 'no rejection' group.*

Biopsy assessment and classification of allograft rejection

All for cause biopsies taken in the first six months after transplantation were re-assessed and classified according to the BANFF 2017 classification.[19,20] Patients empirically treated for rejection without confirmation by a renal biopsy (not performed or no histopathologic changes in the biopsy) were not included in the study (n=14). All diagnoses of 'T cell-mediated rejection' (TCMR) or 'ABMR' were based on histopathologic assessment of a kidney transplant biopsy and serological assessment of DSA, in accordance with the BANFF 2017 classification. For ABMR this consists of histologic evidence of acute tissue injury and of evidence of current/recent antibody interaction with vascular endothelium and of serological evidence for DSA or C4d staining in the biopsy. Cases with ABMR were subsequently subdivided into early (≤14 days) and late (between 15 days and 6 months) rejection. In cases where the biopsy only showed borderline rejection, initiation of treatment for rejection was used to classify the patient as having either TCMR (n=4) or 'no

rejection' (n=4; details in *Supplementary Table 1*). Mixed rejection (ABMR and TCMR) was classified as ABMR (n=1). Some patients had a repeat biopsy. None of the repeat biopsies led to reclassification of the type of rejection.

Baseline characteristics and donation type

Patient, donor and transplantation characteristics and specific risk factors for the development of rejection (including previous transplantations, panel-reactive antibody percentage (PRA), HLA typing, mismatch (HLA-A, HLA-B and HLA-DR), immunosuppressive therapy) were extracted from the electronic health record.

Immunosuppressive regime and induction therapy

Patients received the immunosuppressive regimen according to the standard of care at the time of transplantation. All patients were treated with a combination of a calcineurin inhibitor, prednisolone and mycophenolate mofetil or mycophenolic acid. Before 2001 induction therapy was not part of the standard protocol. In 2001 induction with human interleukin-2 receptor monoclonal antibodies (basiliximab) was introduced, with reduced calcineurin inhibitor exposure. Lymphocyte depleting induction therapy with anti-CD52 monoclonal antibodies (alemtuzumab) was introduced in 2009. The preferred induction therapy depended upon the risk as assessed by the treating physician.

Renal function, patient- and graft survival

Serum creatinine levels at 6 and 12 months after transplantation and information on patient survival and graft survival were obtained from the electronic health record. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI formula.[21] Based on the eGFR, four groups were defined; >50, 30-50 and <30 mL/min/1.73m² and GL (defined as initiation of dialysis as renal replacement therapy).

Assessment of donor specific antibodies

The standard complement dependent cytotoxicity (CDC) test that employs lymphocyte targets to detect complement-fixing IgG and IgM antibodies before transplantation was negative in all patients. A positive CDC test was considered a contra-indication for transplantation. Currently, many transplant centers use a pooled antigen bead (PAB) Luminex assay for the standard work up for a kidney transplantation. In this PAB assay the complete phenotype of class I and II are present on beads and binding of IgG antibodies can be detected by a fluorescence signal. The PAB Luminex assay detects the presence of class I and/or II without specification of the exact antibody. In case of a negative result, absence of antibodies is assumed and further analysis is considered to be redundant. However, in case of a positive result, a single antigen bead (SAB) assay is performed, in order to identify the antibodies and specify donor specificity.

In this cohort both the PAB and SAB assays (regardless of the result of the PAB assay) were performed retrospectively on stored samples of all patients with early ABMR. The samples had been obtained and stored at two timepoints: before transplantation and at the time of rejection. In addition, stored pre-transplant samples of 60 randomly selected patients, who did not develop ABMR, were tested with the SAB assay (15 female recipients with a spousal donor, 15 female recipients with a non-spousal male donor, 15 female recipients with a female donor and 15 male recipients) (*Figure 1*). "The following assays were used. SAB: One Lambda (SA), class 1, catalogue number LSA04NC19_011_00, lot number 3007441, One Lambda (SA), class 2, catalogue number LSA01NC17_012_00, lot number 3007379, LifeCodes (Luminex), class 1, lot number 3008213 and LifeCodes (Luminex), class 2, lot number 3008357. The vendors' protocols and cutoff values were followed"

Statistical analysis

Patient characteristics and risk factors were described as mean ± standard deviation and categorical data as numbers and percentage of the total number. Analysis of differences was performed by Fisher's Exact Test, Independent Samples T-test, and Pearson Chi-square Test. Kidney function was analyzed as a categorical variable. A p-value of <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS version 23.0 (IBM SPSS, Inc., Chicago, IL) and graphs were created with Graphpad Prism version 8.0 (Graphpad Prism Software, Inc., San Diego, CA).

Results

Patient characteristics and the incidence of acute rejection

Baseline characteristics of patients with and without ABMR are summarized in *Table 1*. In the first six months after transplantation, 131 for-cause biopsies were performed in 107 patients. No rejection, TCMR and ABMR was observed in 83% (n=288), 14% (n=53) and 3% (n=11) of patients, respectively. Nine of eleven cases of ABMR were early ABMR and occurred at a median of eight days after transplantation (range 5-14 days). In five of these patients DSA were detected during the rejection episode and eight out of nine patients with early ABMR had C4d positivity in the peritubular capillaries in the biopsy. Two male recipients were diagnosed with late ABMR, 35 and 75 days after transplantation. These patients had de-novo DSA, but no pDSA. All patients with early ABMR were transplanted between 2005 and 2014, none had received a repeat transplant and seven out of nine were non-immunized (i.e. PRA ≤5%). The mean age of the recipient and donor, as well as the degree of HLA mismatch, were not different between patients with and without ABMR. The standard immunosuppressive regimen included steroids, mycophenolate mofetil and a calcineurin inhibitor (either tacrolimus or ciclosporin) and there were no differences between those with and without ABMR (*Table 1* and *Supplementary Table 2*). Patients with ABMR were more likely to have received alemtuzumab as induction therapy. This is most

likely explained by the fact that induction therapy with Alemtuzumab (introduced in 2009) was used more frequently in male to female spousal transplantation, in relation to the clinical perception of an increased risk of early acute rejection.

Table 1. *Patient characteristics stratified by the occurrence of early ABMR (i.e. within two weeks after transplantation) in 352 living unrelated donor kidney transplant recipients, transplanted between 1997-2014 (median 2009, IQR 2006-2012).*

Characteristics	All n = 352	Early ABMR n = 9	No early ABMR n = 343	p-value
Recipient sex - female (%)	143 (41%)	8 (89%)	135 (39%)	<0.01[1]
Recipient age - years (SD)	54 ± 11	55 ± 7	54 ± 11	0.82[2]
Previous transplantation(s) - n (%)	35 (10%)	0	35 (10%)	0.61[1]
Pre-emptive - n (%)	118 (34%)	4 (44%)	114 (33%)	0.49[1]
Donor sex - female (%)	217 (62%)	3 (33%)	214 (62%)	0.09[1]
Donor age - years (SD)	53 ± 11	56 ± 12	53 ± 11	0.45[2]
Mismatch				
HLA A - 0/1/2	31/165/156	0/6/3	31/159/153	0.40[3]
HLA B - 0/1/2	12/125/215	0/3/6	12/122/209	0.83[3]
HLA DR - 0/1/2	22/164/166	0/6/3	22/158/163	0.42[3]
Immunosuppression, induction				
No induction	16 (4%)	0	16 (4%)	
Alemtuzumab	29 (8%)	5 (56%)	24 (7%)	<0.01[3]
Basiliximab	307 (87%)	4 (44%)	303 (88%)	
Immunosuppression, CNI				
Tacrolimus	243 (69%)	5 (56%)	238 (69%)	0.47[1]
Ciclosporin	109 (31%)	4 (44%)	105 (31%)	

Early ABMR = antibody-mediated rejection ≤14 days after transplantation. Pre-emptive = no dialysis treatment before transplantation, CNI = calcineurin inhibiter. [1] Fisher's Exact Test, [2] Independent Samples T-test, [3] Pearson Chi-square Test.

Table 2. *Type of histologically confirmed rejection for the entire cohort in the first six months after transplantation.*

Characteristics	All n = 352	Female recipient male donor spousal n = 61	Female recipient male donor non-spousal n = 36	Female recipient Female donor n = 46	Male recipient n = 209
Early ABMR - *n* (%)	9 (3%)	6 (10%)	0	2 (4%)	1 (<1%)
Late ABMR - *n* (%)	2 (1%)	0	0	0	2 (1%)
TCMR - *n* (%)	53 (15%)	3 (5%)	6 (17%)	4 (9%)	40 (19%)
No rejection[+] - *n* (%)	288 (82%)	52 (85%)	30 (83%)	40 (87%)	166 (79%)

Early ABMR = antibody-mediated rejection ≤14 days after transplantation, late ABMR = antibody-mediated rejection between 15 days and 6 months after transplantation, TCMR = T cell-mediated rejection. [+]No rejection indicates that there was no rejection upon biopsy, or that no biopsy was performed (because there were no clinical signs for rejection).

High incidence of early ABMR in females who received a kidney transplant from their spouse

In order to identify recipients that are particularly at risk for ABMR, the cohort was divided into four groups, based on the recipient sex and donor-recipient relationship (*Table 2*). Overall, 41% of the recipients was female and 43% of these females received a kidney from their male spouse. The pretransplant test for panel reactive antibodies (PRA) was negative in 94% of male recipients and 89% of female recipients. Stratified by type of donor, PRA was negative in 98% of female recipients with a spousal male donor, 81% of female recipients with a non-spousal male donor and 83% of female recipients with a female donor. The fact that nearly all female recipients of a spousal male donor kidney had tested negative in the PRA test before transplantation, reflects clinical practice in which more stringent criteria are applied to these higher risk transplantations.

Table 2 shows that the overall incidence of rejection in the first six months after transplantation was 18% (TCMR 15.1%, early ABMR 2.6%, late ABMR 0.6%). The incidence of TCMR was 19% in male recipients, while only 1% of males developed ABMR. Female recipients, who received a kidney from a male spouse, had a significantly higher incidence of early ABMR compared with the rest of the cohort (10% vs 1%, relative risk 9.5, $p<0.001$), while the incidence of TCMR was 5% in this group. Of note, there were six cases of early ABMR in 61 women who received a kidney from a male spouse, two cases in the other 82 women (relative risk 4.0, $p=0.06$) and only one case in more than 209 men. The low TCMR incidence in female recipients of a spousal donor kidney is most likely due to the choice of induction therapy. After the introduction of alemtuzumab induction therapy in 2009, a higher proportion of female recipients of a spousal donor kidney was treated with alemtuzumab (20/35; 57%), compared with other recipients (9/133; 7%).

Early ABMR leads to a severe reduction in renal function and death censored graft survival

Patient survival was 100% and 99.7% at six and twelve months after transplantation. One patient with polycystic kidney disease, died due to a subarachnoid hemorrhage eight months after transplantation, while having a stable and good kidney function. For the entire cohort, death censored transplant survival in the first year after transplantation was 97%. In the group with early ABMR, this was only 56% (*Figure 2*). Mean eGFR in patients without rejection, TCMR and early ABMR was 57, 47 and 36 ml/min/1.73m^2 respectively, one year after transplantation (in case of a functioning allograft). In the first year GL due to ABMR occurred in four out of nine patients with early ABMR (44%). These patients required dialysis as renal replacement therapy at 9, 11, 96 and 283 days after transplantation. Only one of nine patients with early ABMR reached an eGFR above 50 ml/min/1.73m^2, whereas 69% of those without rejection did so.

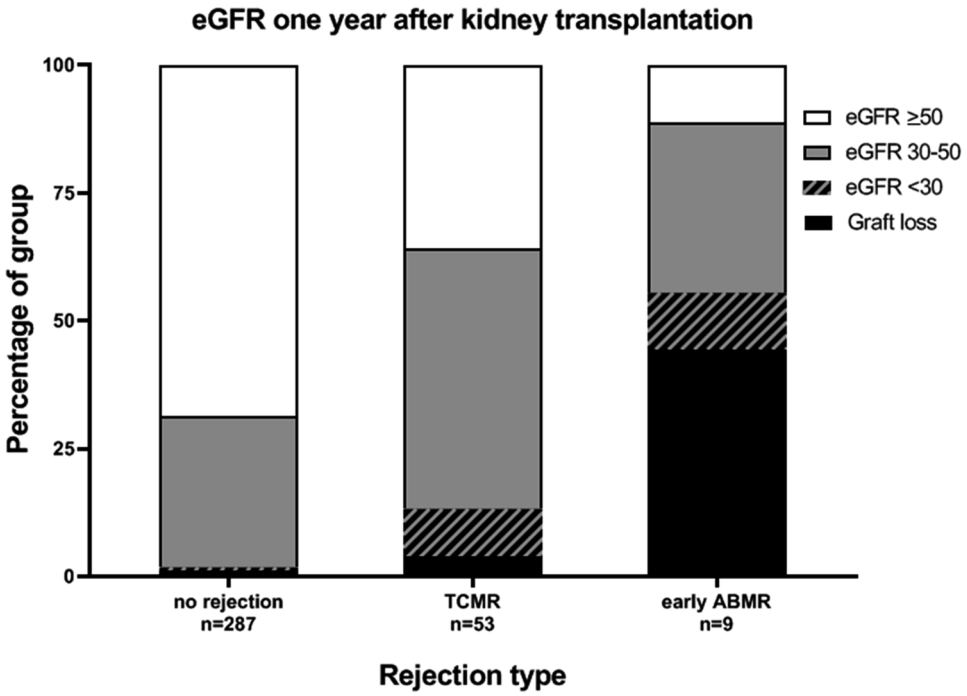

Figure 2. Kidney function one year after transplantation depends on the presence and type of rejection. *56% of patients with early antibody-mediated rejection (ABMR) had kidney graft loss (GL) or an eGFR <30 ml/min/1.73m^2 within 1 year after transplantation. One of the patients with early ABMR reached a kidney function >50 ml/min/1.73m^2, while 69% of patients without rejection reached this level. One patient without rejection died at eight months. Two patients with late ABMR (between 15 days and 6 months after transplantation) are not depicted (resp. eGFR 17 and 51 ml/min/1.73m^2).*

Table 3*. Association between early ABMR and history of pre-transplant blood transfusions and pregnancies in women who received a kidney from an unrelated donor.*

Characteristics	All n = 93	Early ABMR n = 7	No ABMR n = 86	Univariate OR (95% CI)
Blood transfusions				
None	40	4 (57%)	36 (42%)	
≥ 1	48	2 (29%)	46 (53%)	0.4 (0.1-2.3)
Unknown	5	1 (14%)	4 (5%)	
Pregnancies				
None	16	1 (14%)	15 (17%)	
≥ 1	76	6 (86%)	70 (81%)	1.4 (0.2-12.2)
Unknown	1	0	1 (1%)	

Missing data: one in the 'early ABMR' group, 49 in the 'No ABMR' group. Early ABMR = antibody-mediated rejection ≤14 days after transplantation.

Blood transfusions and pregnancies did not correlate with ABMR

In order to clarify the role of immunizing events among female recipients, we analyzed the association between pre-transplant blood transfusions and pregnancies and the development of ABMR. Data was complete for 65% (93/143) of female recipients, for 67% (41/61) of female recipients who received a transplant from their spouse and for seven out of eight female recipients with ABMR. The prevalence of blood transfusions before transplantation and pregnancies did not differ between women with or without ABMR, as shown in *Table 3*. Furthermore, in the subset of women who received a kidney from their spouse, the percentage of women who had been pregnant with the donor's child was no different in those with ABMR (67%, 4/6) than in those without ABMR (66%, 23/35).

Patients with early ABMR had preformed DSA in spite of having tested negative in pre-transplantation CDC test and pooled antigen bead assay

As described above, the median time to early ABMR after transplantation was eight days (range 5-14). Such an early onset of a humoral response is a strong indication that ABMR was caused by pDSA. We retrospectively performed testing for pDSA, using a SAB assay. Analysis of the cases in which ABMR occurred within two weeks after transplantation, showed that seven out of nine patients (78%) had pre-formed class I and/or II anti-HLA DSA (*Table 4*). In four of these patients, pDSA were only detected by using the SAB assay, but not in the PAB assay. pDSA were HLA class I in five patients, class II in one patient and both class I and II in one patient. The median MFI was 2200 (IQR 1400-2700, range 700-5500). pDSA were found in all three patients with GL within the first six months after transplantation. Only one of them had a positive PAB assay, while all three had pDSA in the SAB assay. At the time of rejection, five out of nine patients with early ABMR had DSA (class I in two patients, class II in one patient and class I and II in two patients).

In order to assess the prevalence and clinical significance of a positive single antigen test before transplantation, we performed a SAB assay in 60 randomly selected patients without signs of ABMR (*Figure 1*). The test yielded pDSA in four patients (7%). Of 15 female recipients without ABMR and with a spousal donor, one had pDSA. In comparison, pDSA were detected in five of six female recipients with ABMR and with a spousal donor, resulting in a specificity of 93% and sensitivity of 83% of pDSA for early ABMR within this group of recipients.

Table 4. *Patient characteristics, presence of (p)DSA and kidney function for nine renal recipients with early ABMR.*

Recipient sex	Donor type	Initial kidney disease	Risk factors before Tx	Induction therapy	ABMR days after Tx	DSA (MFI) before Tx	DSA (MFI) during ABMR	eGFR Tx + 1y
female	male spousal	MPGN	reTx: - preg: + PRA: - btf: -	Amab	7	A1 (900), DQ2 (1,900)	DQ2 (3,100)	GL
female	male spousal	PKD	reTx: - preg: + PRA: - btf: -	Amab	8	B51 (2,700) *	B51(2,200), B38(2,200), DR5 (3,400)	GL
female	male spousal	secondary hyperoxaluria	reTx: - preg: + PRA: + btf: +	Amab	14	A2 (2,300), B57 (2,800) *	A2 (7,000), B44 (8,300)	GL
female	male spousal	hypertension	reTx: - preg: + PRA: - btf: +	Amab	11	B58 (730) *	neg	26
female	male spousal	Secondary FSGS	reTx: - preg: - PRA: - btf: -	Bmab	5	DQ2 (2,200) *	neg	59
female	male spousal	diabetic nephropathy	reTx: - preg: + PRA: - btf: -	Amab	7	neg	neg	47
female	female	FSGS	reTx: - preg: + PRA: - btf: u	Bmab	7	A2 (5,500)	A2 (15,300), A3 (14,200)	45
female	female	PKD	reTx: - preg: + PRA: + btf: -	Bmab	12	neg	neg	GL
male	female	unknown cause	reTx: - preg: PRA: - btf: u	Bmab	8	B7 (1,900)	B7 (15,300), DR13 (20,400), DR52 (11,000)	40

*The pre-transplantation complement dependent cytotoxicity test was negative in all patients. The PRA was positive in two patients (26% and 36%). Early ABMR = antibody-mediated rejection ≤14 days after transplantation, Tx = transplantation, DSA = donor specific antibodies, MFI = Mean Fluorescence index, eGFR in ml/min/1.73m2, MPGN = membranoproliferative glomerulonephritis, PKD = polycystic kidney disease, FSGS = focal segmental glomerulosclerosis, SLE = systemic lupus erythematosus. reTx = repeat transplant, PRA = panel-reactive antibody (<5% considered negative), preg = pregnancy, btf = blood transfusion, u = unknown. Amab = Alemtuzumab. Bmab = Basiliximab. GL = graft loss and was due to ABMR. *solely detected by single antigen bead assay.*

Discussion

We found that early ABMR occurred in one in ten women who received a kidney transplant from a male spouse, with detrimental consequences to graft function. The incidence of early ABMR was 2% in other women and <1% in men. Furthermore, we show that in the pre-transplant assessment of women who receive a kidney transplant from their male spouse, even when the PAB is negative a SAB assay should be performed to lower the risk of early ABMR. The risk of developing early ABMR could not be predicted by assessing classical clinical patient characteristics, such as prior blood transfusion or pregnancy.

The median time from transplantation to ABMR was very short (eight days in early ABMR). Therefore, it is not surprising that in retrospect, pDSA were present in 78% of cases with early ABMR. In general, de-novo DSA would take more time to develop and are not likely to be formed so early, particularly given the current potent immunosuppressive drug regimen.

In our cohort, early ABMR only occurred in recipients of a living donor kidney transplanted after 2004. Most likely, this reflects a change in practice through time. Firstly, living kidney donation has become more common. Secondly, with the availability of more sensitive assays to screen for pDSA and the advent of stronger immunosuppressive drugs, male to female spousal transplantations, which traditionally have been viewed as carrying a higher immunological risk and were often avoided, were deemed safe. It is important to note that in spite of testing for pDSA with a combination of assays (CDC test and PAB assay) in an experienced, specialized laboratory, pDSA can go undetected. The SAB assay revealed pDSA in up to 57% of patients with early ABMR, despite a negative PAB assay.

In accordance with previous studies, patients with ABMR had an inferior outcome in terms of eGFR and/or graft loss in the first year after transplantation, compared with recipients without ABMR or pDSA.[22] Furthermore our study corroborates the fact that presence of pDSA, despite a negative CDC test, is a key parameter, indicating a strong increase in the risk of early ABMR.[23] Other studies show that especially pDSA that persist after transplantation cause ABMR and a worse outcome, while recipients with pDSA that disappear after transplantation tend to have the same outcome as recipients without pDSA.[24]

In our cohort, prior pregnancies were as common in women with, as in women without early ABMR. Nevertheless, in the literature there are several indications that a proportion of renal recipients develop DSA due to sensitization by a previous pregnancy. After a failed previous transplant, pregnancy is considered to be the second most prominent immunizing event.[25] Terasaki reported that in spousal donations, females who had been pregnant before transplantation tended to have a worse three-year graft survival than female recipients without pregnancies.[26] This type of immunization is a risk factor for the development of DSA[27] and early graft loss, in particular if the mismatch with the donor

kidney is repeated in the HLA profile of the father of the child.[28,29] In addition, a higher rate of hyperacute rejection and GL has been observed in spousal male to female donations in general, compared with living related donations.[30] With respect to the type of rejection, ABMR has been reported to be more frequent in spousal kidney transplantation than in living related kidney transplantation, in particular in patients with a low-risk pre-transplant risk profile for ABMR.[15,31] In the majority of these cases, changes in kidney function are not reported. Despite these risks, spousal LURD kidney donation is generally considered to be relatively safe, compared with other living kidney donations. Our study adds data showing that this is not the case and that additional care is needed to safely conduct male to female spousal LURD.

In contrast with other studies, we did not find a higher incidence of ABMR in patients who received blood transfusions before transplantation. It is reported that especially blood transfusions that share HLA antigens with the allograft are a risk factor for the development of transfusion specific antibodies that may harm the allograft.[32] This discrepancy between the literature and our results may be related to the fact that blood transfusion products are entirely leukocyte depleted since 2001 which significantly reduces the immunological risk.

This study has a number of strengths. First, the cohort was large and well defined and focused both on clinical and immunological risk factors for ABMR. Second, biopsies were assessed by an experienced nephropathologist and classified using the most recent guideline (BANFF 2017 classification[33]). Furthermore, extensive DSA testing was performed by a Eurotransplant reference laboratory. Last, we assessed the prevalence of pDSA in a random selection of recipients without ABMR, to gain insight into the occurrence and relevance of pDSA that are detected with the SAB assay, while not detected by the PAB assay.

Our study also has limitations. Firstly, despite it being a large cohort, the overall incidence of early ABMR was low; nine cases in total. Based on our results, we can confidently state that screening for pDSA with a PAB assay suffices for male transplant recipients and that screening with a SAB assay should be included in the pre-transplant assessment of women who are to receive a kidney from their spouse. Uncertainty remains regarding other female recipients. In our study, only women with a male spousal donor had pDSA that were not detected with a PAB assay. Therefore, we limit the recommendation to include a SAB assay in the pre-transplant work-up to women who are to receive a kidney from a spouse. Secondly, we did not test C1q binding by DSA. Since DSA that bind C1q are associated with an increased risk of ABMR and a higher risk of graft loss,[34-36] this test could be of value in discerning relevant from irrelevant pDSA. However, the increased risk of ABMR is described for de-novo DSA in particular and there is no consensus about the relevance of C1q binding for clinical outcomes.[37,38] The same applies to the relation between DSA and the role of T-cells and NK cells in ABMR.[39] Furthermore, information on previous blood transfusions and pregnancies was incomplete. The results of our analysis, however, do suggest that it is

unlikely that these clinical characteristics have a reliable predictive value for early ABMR. Lastly, female recipients who received a kidney from their spouse were more often treated with alemtuzumab as induction therapy and this may have lowered the incidence of ABMR in this specific group. In fact, 25% of female recipients with a spousal donor developed early ABMR, despite having received alemtuzumab induction therapy.

We conclude that risk assessment for ABMR benefits from the addition of the SAB assay in all female recipients of a spousal donor kidney transplant. We observed a high incidence of ABMR in this subgroup as well as a significantly inferior outcome in terms of eGFR and graft survival. A positive SAB in this group, should encourage the option of indirect (cross-over) donation.

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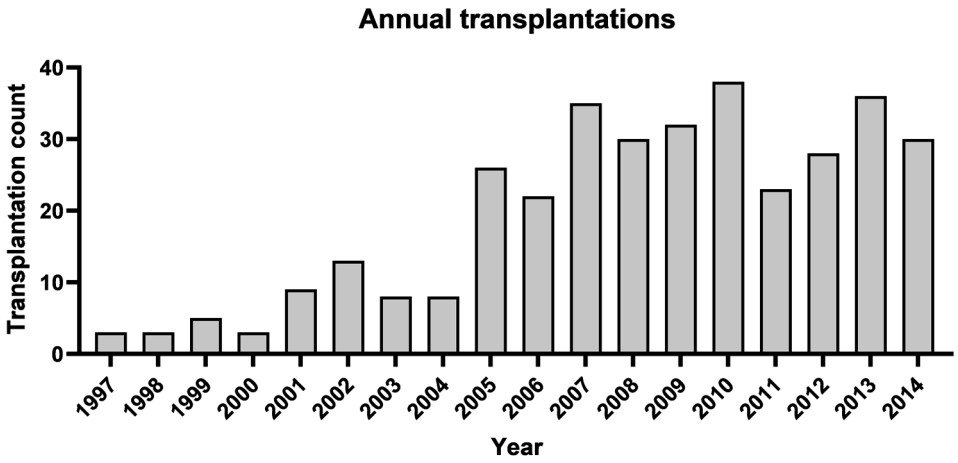
Supplementary information



Supplementary Figure 1. *The year of transplantation of the 352 individuals in the cohort.*

Supplementary Table 1. *Patients with borderline rejection that are classified as 'TCMR' or 'no rejection', based on clinical treatment.*

Donation type (age recipient)	Initial disease	Biopsy after Tx (days)	eGFR at 1Y after Tx (ml/min/1.73m^2)	Year of Tx
Borderline rejection classified as TCMR				
Female to male (60Y)	MPGN	8	42	1999
Female to male (65Y)	Unknown	8	35	2007
Female to male (60Y)	Unknown	10	36	2013
Male to male (39Y)	Unknown	3	47	2013
Borderline rejection classified as No rejection				
Female to female (68Y)	IgA	145	43	2005
Female to female (70Y)	Hypertension	164	39	2010
Female to female (67Y)	Hypertension	12	77	2008
Female to male (55Y)	Diabetes	70	30	2008

Tx = transplantation, MPGN = membranoproliferative glomerulonephritis.

Supplementary Table 2. *Induction therapy and calcineurin inhibitor in donation groups.*

	All	Female recipient male donor		Female recipient Female donor	Male recipient
		spousal	non-spousal		
Early ABMR	n = 9	n = 6	n = 0	n = 2	n = 1
Induction – *n* (%)					
No induction	-	-	-	-	-
Alemtuzumab	5 (56%)	5 (83%)	-	-	-
Basiliximab	4 (44%)	1 (17%)	-	2 (100%)	1 (%)
CNI – *n* (%)					
Tacrolimus	5 (56%)	5 (83%)	-	-	-
Ciclosporin	4 (44%)	1 (17%)	-	2 (100%)	1 (%)
No ABMR	n = 343	n = 55	n = 36	n = 44	n = 208
Induction – *n* (%)					
No induction	16 (5%)	4 (7%)	-	1 (2%)	11 (5%)
Alemtuzumab	24 (7%)	15 (27%)	4 (11%)	2 (5%)	3 (1%)
Basiliximab	303 (88%)	36 (65%)	32 (89%)	41 (93%)	194 (93%)
CNI – *n* (%)					
Tacrolimus	238 (69%)	39 (71%)	23 (64%)	32 (73%)	144 (69%)
Ciclosporin	105 (31%)	16 (29%)	13 (36%)	12 (27%)	64 (31%)

Next to a calcineurin inhibitor, all patients were treated with prednisolone and either mycophenolate mofetil or mycophenolic acid. Early ABMR = antibody-mediated rejection ≤14 days after transplantation, CNI = calcineurin inhibitor.

Chapter 6

Autologous bone marrow derived mesenchymal stromal cell therapy with early tacrolimus withdrawal: the randomized, prospective, single-center, open-label, TRITON study

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Abstract

Background

After renal transplantation, there is a need for immunosuppressive regimens which effectively prevent allograft rejection, while preserving renal function and minimizing side effects. From this perspective, mesenchymal stromal cell (MSC) therapy is of interest.

Methods

In this randomized prospective, single-center, open-label trial, we compared MSCs infused 6 and 7 weeks after renal transplantation and early tacrolimus withdrawal with a control tacrolimus group. Primary end point was quantitative evaluation of interstitial fibrosis in protocol biopsies at 4 and 24 weeks posttransplant. Secondary end points included acute rejection, graft loss, death, renal function, adverse events, and immunological responses.

Results

Seventy patients were randomly assigned of which 57 patients were included in the final analysis (29 MSC; 28 controls). Quantitative progression of fibrosis failed to show benefit in the MSC group and GFR remained stable in both groups. One acute rejection was documented (MSC group), while subclinical rejection in week 24 protocol biopsies occurred in seven patients (four MSC; three controls). In the MSC group, regulatory T cell numbers were significantly higher compared to controls (p=0.014, week 24).

Conclusions

In conclusion, early tacrolimus withdrawal with MSC therapy was safe and feasible without increased rejection and with preserved renal function. MSC therapy is a potentially useful approach after renal transplantation.

Introduction

Over the last two decades significant progress has been achieved in short-term survival of kidney transplants.[1,2] Unfortunately, these advancements have not led to a similar improvement in long-term kidney transplant survival rates. Various factors, including donor graft quality, ischemia/reperfusion (I/R) injury, alloreactivity, viral infections, and drug therapy, may adversely affect renal structure causing graft scarring and compromising long-term function.[3] The intensity of current immunosuppressive drugs, albeit efficacious in preventing rejection, is associated with increased risk for (viral) infections and malignancies. Calcineurin inhibitors (CNIs) are the cornerstone of current immunosuppressive therapy, but they have direct nephrotoxic effects. It has been demonstrated that CNI withdrawal should be undertaken before month 6 to prevent the occurrence of irreversible tubulointerstitial damage.[4,5] So far, early CNI withdrawal studies have proven to be risky and invariably lead to increased rejection and even loss of grafts.[6] Consequently, there is a need for immunosuppressive regimens that can prevent allograft rejection, while preserving renal function and promoting patient and graft survival in the long term. MSCs have immunosuppressive properties and roles in tissue repair, and various (mainly experimental) studies have demonstrated that MSCs may increase regulatory T cell (Treg) levels and polarize the immune system toward tolerance.[7,8] In renal transplantation, early studies using MSCs focused on safety and feasibility.[9-12] Although most of these studies were not designed as efficacy trials, there were indications that MSCs possess immunosuppressive properties, as evidenced by an increase in Tregs and downregulation of cytotoxic $CD8T^{+}$ cells in a small number of patients. We performed a randomized, prospective, single-center, open-label study in living-donor kidney transplant recipients in which we compared autologous bone marrow (BM)-derived MSC therapy (infused at weeks 6 and 7) with concomitant early tacrolimus withdrawal (at week 8) to standard tacrolimus dose. Primary end point was quantitative evaluation of interstitial fibrosis and secondary end points included biopsy-proven acute rejection, graft loss, death, renal function, adverse events, and immunological responses at week 24. We chose to perform the study on a background of alemtuzumab-based induction to minimize the risk for acute rejection[13] and mTOR inhibition, since experimental studies demonstrated tolerogenic properties in combination with MSCs.[14] In a post hoc long-term analysis, peripheral blood immune cell composition was also obtained at week 52 in patients with sufficient follow-up. In addition, the efficacy end point (biopsy-proven acute rejection (BPAR), graft loss, or death) was obtained up to 5 years in patients who had a longer follow-up.

Materials and methods

Study design and patients

The TRITON study is a 24-weeks investigator-initiated, randomized, prospective, open-label, single-center, clinical study, performed at the Leiden University Medical Center (LUMC), the Netherlands. The trial design has been published previously.[15] The trial protocol, available at the Appendix S1 and S2 section, was approved by the local ethics committee at the LUMC, Leiden, and by the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. The trial was performed in accordance with the principles of the Declaration of Helsinki. In total, 70 de novo renal recipients of a kidney from a living donor, 18–75 years of age, were recruited from the transplant clinics of the LUMC. The inclusion/exclusion criteria were described previously.[15] Written informed consent was obtained from all participants.

Randomization and masking

Patients were randomly assigned before transplantation to either the MSC or control group in a ratio 1:1 (*Figure S1*). A patient was randomized only after verification of eligibility and informed consent. The randomization procedure was designed and implemented by the IMO (Informatie Management Onderzoek) department of the University Medical Center Groningen (UMCG), the Netherlands, using a web-based system (ALEA). Investigator or authorized delegate from the study staff received an individual login code with which they could randomize their patients. The web application returned the allocated treatment. As a confirmation, the web application also sent an e-mail with the randomization information to selected users. Patients maintained this randomization number throughout the study. Because of the nature of the intervention (BM biopsy and MSC infusions), participants and physicians were not masked to treatment assignment.

Procedures

All patients in the study received alemtuzumab (anti-CD52),15 mg subcutaneously, at days 0 and 1 as well as tacrolimus (Prograft®), everolimus (EVL; Certican®), and low-dose prednisone, as maintenance therapy (*Figure S1*).[15] Patients in the MSC group received two doses of autologous BM MSCs, intravenously at weeks 6 and 7 after transplantation. Autologous MSCs were chosen instead of third-party MSCs to prevent alloimmunization. The dose of tacrolimus was reduced to 50% at the time of the second MSC infusion and completely withdrawn 1 week later. Patients received a higher dose of prednisolone (15 mg instead of 10 mg) for 14 days after the second infusion to diminish risks of tacrolimus withdrawal. In patients in the control group, the trough level of tacrolimus was lowered to a target of 6–8 ng/ml 8 weeks after transplantation. BM was aspirated from the posterior iliac crest of all patients in the MSC group under general anesthesia during the renal transplantation, as described previously.[15] This protocol was approved by the local ethics

committee (P13.283) and by the CCMO (NL4371200013). Processing of the MSCs took place at the Interdivisional Good Manufacturing Practice (GMP) Facility of the LUMC (*Table S1*).[15] The MSC product was infused via peripheral infusion within 30 min with a target dose of 1.5×10^6 per/kg body weight IV (range $1–2 \times 10^6$), according to our previous study.[15] Monitoring of the patients occurred according to the assessment schedule, as described in the protocol (page 28).

Outcomes

The primary end point was the quantitative progression of interstitial fibrosis between the 4- and 24-week protocol biopsies as measured by morphometric analysis of collagen deposition. Interstitial collagen fibers in protocol biopsies were visualized by Sirius Red (SR) staining and quantified as a percentage of total tubulointerstitial tissue (glomeruli and large vessels excluded) by quantifying positive pixels in five representative locations at 40× magnification with a macro created in ImageJ version 1.50i.[16] Included secondary end points were composite end point efficacy failure (BPAR, graft loss, or death); proteinuria, Banff scores at the protocol biopsies, renal function as measured by estimated (e) glomerular filtration rate (GFR), (serious) adverse events ((S)AE), including (viral) infections, the presence of de novo donor-specific antibodies (dnDSA), and peripheral blood immune cell composition. Scoring of renal biopsies was performed in a blinded fashion by a renal pathologist from our center after completion of the study, using the most recent Banff classification.[17] Findings in a protocol biopsy with evidence of rejection were reported as subclinical acute rejection (SCAR). Renal function was calculated by the eGFR (ml/min/1.73 m^2) using the CKD-EPI formula.[15] AEs and SAEs were documented according to Medical Dictionary for Regulatory Activities (MedDRA®); the international medical terminology developed under the auspices of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. Tacrolimus and EVL quantification was assessed using a previously validated LC–MS/MS assay.[18]

Immunological monitoring

For human leukocyte antigen (HLA) antibody analysis, serum samples were screened using Luminex screen assay (Lifecodes, Immucor) and analyzed with a Luminex 200 reader. Definitions of the negative/positive discriminations were used as suggested by the provider. When positive, a single antigen bead (SAB) assay (Lifecodes, Immucor) was performed as standard-of-care. Assignment of positivity was assessed according to the manufacturer's instructions. Since MSCs are suggested to have immunomodulatory properties, we performed phenotypical analysis of leukocyte subpopulations on fresh whole blood. Staining, acquisition, and data analysis were performed strictly adhering to “The One” study protocol.[19] Absolute cell counts were obtained using the BD Multitest kit (BD Biosciences).

Post hoc analysis

Phenotypical analysis of leukocyte subpopulations was, in addition to the 24-week time point, also performed 52 weeks after renal transplantation. Assessment of composite end point efficacy failure (BPAR, graft loss, or death) and renal function by eGFR was also obtained in patients with a follow-up up to 5 years in a post hoc analysis (n = 52 at 1 year, n = 40 at 2 years, n = 24 at 3 years, n = 17 at 4 years, and n = 13 at 5 years, *Table 4*).

Statistical analysis

The study was designed to have a sample size of 25 in each group, or 50 in total, to have a power to detect a relative difference in mean percentages of fibrosis of at least 25% using an independent sample t test with a 0.05 two-sided significance level (α), as described previously.[15] We anticipated that 70% of the included patients would have valid measurements (withdrawal included) and therefore included 70 patients. Data analysis was performed using SPSS version 25.0 (SPSS, Inc.) and all graphs were created using GraphPad Prism version 8.0 (GraphPad Prism Software, Inc.). Parametric data were described as mean ± SD, nonparametric data as median and interquartile range (IQR), and categorical data as numbers and percentages. $p<0.05$ were considered statistically significant. The slopes of eGFR data were calculated and analyzed using a linear regression analysis. Immune monitoring data were analyzed using the Mann–Whitney test with Bonferroni correction for multiple testing. A data safety monitoring board (DSMB) monitored the safety of subjects. The trial is registered with ClinicalTrials.gov, NCT02057965.

Results

Patients

Between March 3, 2014 and January 17, 2020, 70 patients, aged 19 to 74 years, were enrolled in the study: 36 patients were randomly assigned to the MSC group and 34 to the control group (*Figure 1*). Thirteen patients did not receive allocated treatment, because of abnormal MSC growth (defined as karyotypic abnormalities in the final product; *n*=4), contra indication for MSC infusion due to the COVID-19 pandemic (*n*=1), impossibility of obtaining a baseline renal biopsy (*n*=2 in MSC and *n*=1 in control group), withdrawn informed consent (*n*=4 in control group) and (relative) contra indication for prednisone usage (*n*=1 control group). In total, 29 patients were assigned to the MSC and 28 to the control group (*Figure 1*). Patient baseline characteristics were similar in both groups (*Table 1*). Of the 29 patients in the MSC group, 28 patients received two infusions of MSCs, all within the proposed range. One patient received one dose of MSCs within the proposed range. The second dose was not given because of the COVID-19 pandemic. This patient gave informed consent to continue the study. All patients had stable vital signs before and after MSC infusion monitored using MEWS (*Table S1*). In 28 patients in the MSC group and 23

patients in the control group, two renal biopsies could be obtained (*Figure 1*), in order to assess the quantitative progression of interstitial fibrosis.

Quantitative progression of fibrosis score

The quantitative progression of fibrosis score in the biopsies was similar in both groups (MSC group 1.0 ± 7.9; control group 0.3 ± 7.8, *p*=0.755). The fibrosis score remained stable both within the MSC (week 4, 15.2 ± 6.6 and week 24, 16.2 ± 5.3, *p*=0.526) and control group (week 4, 17.0 ± 4.6 and week 24 17.3 ± 5.7, *p*=0.870) (*Figure 2*; *Figure S2*). Delta Banff scores from 4 to 24 weeks were similar in the two groups, in particular the delta ti-score (*p*=0.8), the delta interstitial fibrosis/tubular atrophy (IFTA) score (*p*=0.4), and the delta ah-score (*p* = 0.4) (*Figure S3*).

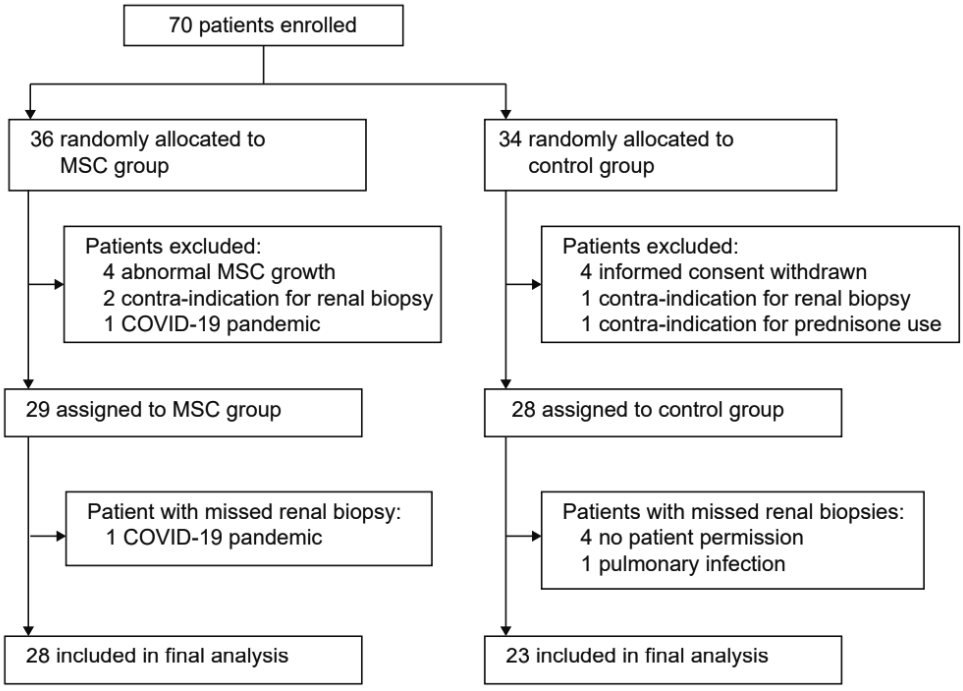

Figure 1. Trial profile. *MSC = mesenchymal stromal cell.*

Table 1. *Baseline Characteristics.*

Characteristic	MSC group (n=29)	Control group (n=28)
Recipient		
Age, mean (SD), yr	50 (14%)	50 (15%)
Male sex, no. (%)	26 (90%)	20 (71%)
Body weight, mean (SD), kg	81 (14%)	82 (14%)
Primary diagnosis, no. (%)		
IgA nephropathy	7 (24%)	3 (11%)
Hypertension	3 (10%)	9 (32%)
Polycystic kidney disease	9 (31%)	3 (11%)
Diabetes	5 (17%)	0
Reflux nephropathy	0	2 (7%)
Membranous nephropathy	1 (3%)	1 (4%)
Lupus nephritis	1 (3%)	0
Other	2 (7%)	3 (11%)
Unknown	1 (3%)	7 (25%)
Donor		
Age, mean (SD), yr	55 (13%)	51 (11%)
Male sex, no. (%)	14 (48%)	10 (36%)
eGFR (pre-donation), mean (SD)	109.7 (12.0)	109.3 (12.7)
Transplant		
Type, related, no. (%)	13 (45%)	15 (54%)
HLA A/B mismatch, mean (SD)	2.3 (1.3)	2.4 (0.9)
HLA DQ/DR mismatch, mean (SD)	1.2 (0.6)	1.3 (0.5)
Cold-ischemia time, mean (SD), hr	3.1 (0.6)	3.0 (0.5)
1st warm ischemia time, mean (SD), min	3.7 (2.1)	5.2 (4.3)
2nd warm ischemia time, mean (SD),	27.0 (3.7)	31.1 (14.4)
Cytomegalovirus IgG status, no. (%)		
D+ / R+	9 (31%)	6 (21%)
D+ / R-	7 (24%)	9 (32%)
D- / R+	1 (3%)	2 (7%)
D- / R-	12 (41%)	11 (39%)
Epstein-Barr virus IgG D+/R, no. (%)	1 (3%)	1 (4%)

GFR = glomerular filtration rate, HLA = Human Leukocyte Antigen. All data are described as mean (SD) or No. (%) (mentioned in every specific variable row).

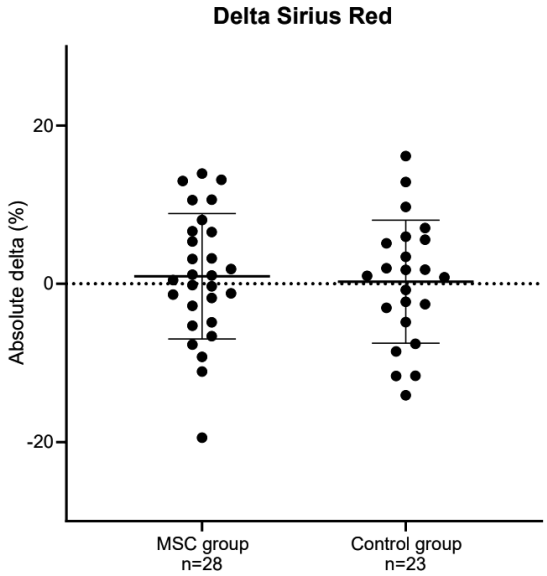

Figure 2. Interstitial fibrosis scores. *Quantitative progression of interstitial fibrosis (delta Sirius Red) between the 4 and 24-week renal biopsy (percentage). MSC = mesenchymal stromal cell.*

Table 2. *Secondary endpoints (graft loss, renal function, biopsy scores) during the study period of 24 weeks.*

Endpoint study period of 24 weeks	**MSC group (n=29)**	**Control group (n=28)**
Graft loss, No. (%)	0	0
eGFR <30ml/min/1.73m2, No. (%)	0	3 (12%)
Patients with for-cause biopsies, No. (%)	1 (3%)	4 (14%)
ABMR, TCMR II and BK nephropathy	1	
BK nephropathy		1
Acute tubular necrosis		1
Hyaline thickening		1
No abnormalities		1
4 wks	29 (100%)	28 (100%)
ABMR	1	0
No rejection	28	28
24 wks	28 (97%)	23 (82%)
TCMR IA	1	2
ABMR	2[a]	0
ABMR and TCMR IA	1	1
No rejection	24	20

MSC = mesenchymal stromal cell; eGFR = estimated glomerular filtration rate; ABMR = antibody mediated rejection; TCMR = T-cell mediated rejection; TIN = tubulointerstitial nephritis; IFTA = interstitial fibrosis and tubular atrophy. All data are described as No (%) (also mentioned in every specific variable row). [a] one patient demonstrated ABMR at 4 and 24 weeks.

Patient survival, renal function, and biopsy scores

Patient survival during the study follow-up was 100% in both groups. All patients had a functioning kidney graft at the end of the 24-week study period (*Table 2*). eGFR was 56 ± 16 ml/min/1.73 m^2 in the MSC (*n*=29) and 42 ± 9 ml/min/1.73 m^2 in the control group (*n*=28) at the time of MSC infusion (*Figure 3A*). Mean eGFR and 24-h proteinuria (*Table S2*) in the MSC group were similar as compared with the control group, with a mean of 56 ± 15 ml/min/1.73 m^2 and 47 ± 16 ml/min/1.73 m^2, respectively, at week 24 (*Figure 3A*). The slope from 4 to 24 weeks in the MSC group (slope=−0.22; intercept=58.15) was not significantly different from the control group (slope=0.09; intercept=43.33) (*p*=0.08, *Figure 3B*). Only one acute rejection episode (combination of T cell [TCMR] and antibody-mediated rejection [ABMR]), documented by for-cause biopsy, was found during the study period in the MSC group (1/29 or 3.4%) (*Table 2*). In this patient, immune suppression had been further reduced due to persistent BK viremia/nephropathy. In the control group, four patients had an indication for a for-cause renal biopsy, without evidence of rejection (*Table 2*). The 24-week protocol biopsies showed SCAR in 14.3% and 13.0% of patients in the MSC (4/28) and control group (3/23), respectively. Protocol biopsies in the MSC group showed a chronic active TCMR Banff IA (*n*=1 patient), active ABMR (*n*=2, of which one also had active ABMR in the 4-week protocol biopsy; both having class I and II DSAs, C4d positive only at 6 months), and one mixed active ABMR and acute TCMR IA. Biopsies in the control group demonstrated acute TCMR Banff IA (*n*=2 patients) and a mixed active ABMR and acute TCMR IA (*n*=1 patient) (*Table 2*). All patients had a negative HLA antibody screening before and 4 weeks after transplantation. In the MSC group, seven patients developed dnDSA at week 24 (24%) (*Table 3*). Their protocol renal biopsies demonstrated no rejection (*n*=3), borderline suspicious for acute TCMR (*n*=1), ABMR (*n*=2, both C4d negative), and ABMR/TCMR IA (*n*=1, $C4d^+$). In the control group, two patients developed HLA class-II dnDSA without signs of rejection in their protocol biopsies.

Immunosuppressive drug levels and change of regime

Immunosuppressive drug levels were within or only slightly out of prespecified target ranges. EVL levels, however, were significantly lower at three time points in the control group (*Table S3*). All patients in the MSC group were on EVL at the end of the 24-week study period. In the MSC group, tacrolimus was reintroduced in one patient, because of acute rejection. In the control group, tacrolimus was discontinued in two patients because of BK nephropathy. EVL was switched to mycophenolate mofetil in four patients after a thrombovascular event and discontinued in two patients (CMV infection and infected lymphocele, respectively).

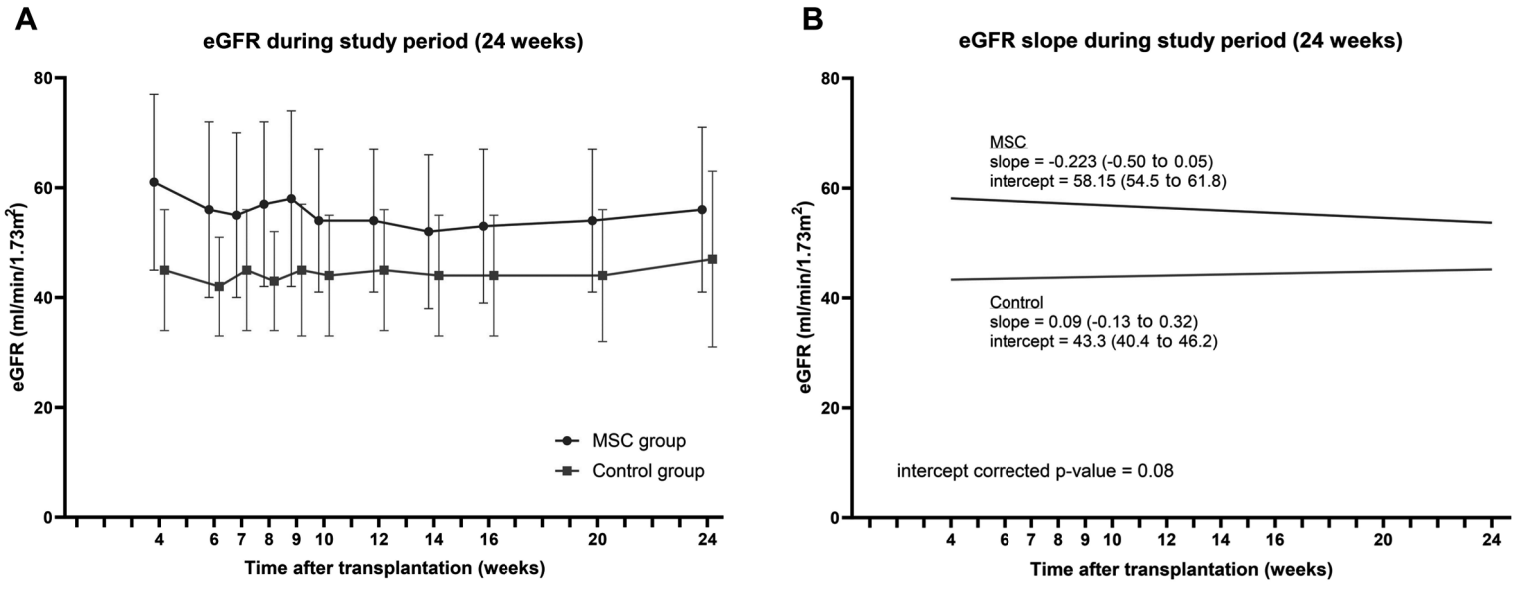

Figure 3. eGFR during the study period of 24 weeks. *(A) eGFR (ml/min/1.73 m²), calculated by the CKD-EPI formula and depicted per time point as mean ± SD, of patients in the MSC and control groups. (B) Slopes of the eGFR in the MSC group were not significantly different from the control group (p = 0.08). Slope and intercept data per group are described, including 95% confidence intervals.*

(Serious) adverse events

Forty-four SAEs were reported, of which 19 in the MSC and 25 in the control group. In total, 272 AEs were reported in the MSC and 301 in the control group (*Table 3*). There were no AEs directly related to the MSC infusions. In the control group, 15 viral infections (EBV, CMV, and BK viremia) developed and 14 in the MSC group (*Table 3*). BK nephropathy occurred in one patient in the MSC (3%) and in three patients in the control group (11%).

Immune monitoring

Immune monitoring studies demonstrated that absolute numbers of peripheral blood $CD45^+$ leukocytes and $CD14^+$ monocytes remain stable after transplantation between weeks 6 and 52 in the MSC and control groups (*Figure 4A,B*). $CD19^+$ B cells and $CD56^+$ NK cells decreased after alemtuzumab-based induction in both groups and re-appeared from week 12 onwards; however, no statistically significant change was measured between the groups (*Figure 4C,D*). $CD3^+CD8^+$ T cells, $CD3^+CD4^+$ T cells, as well as $CD4^+CD25^{hi}CD127^{lo}$ Tregs showed a decrease after alemtuzumab-based induction in both groups while still being suppressed at week 52 (*Figure 4E,G*). Total Treg numbers were significantly higher in the MSC group with tacrolimus withdrawal as compared to the control group at 24 and 52 weeks after transplantation (p=0.014 and p=0.047, respectively), due to the increase in absolute number of $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ memory Tregs (p=0.040 and p=0.047) (*Figure 4G,H*). Absolute numbers of naïve Tregs ($CD4^+CD25^{hi}CD127^{lo}CD45RA^+$) were similar in both groups (*Figure S4*). Percentages of total and naïve Tregs were not different between the two groups at any time points, whereas percentages of memory Tregs within the total CD4 population were elevated in the control group only at week 12, which normalized the weeks thereafter (*Figure S5*).

Post hoc analysis

In the post hoc longer (intermediate)-term follow-up analysis (up to 5 years), graft loss was observed in two patients in the control group (*Table 4*). Renal function in the MSC group was preserved with an eGFR between 47 and 57 ml/min/1.73 m^2 (*Table 4*). In the patients in the control group, eGFR gradually declined with a mean of 42 ml/min/1.73 m^2 at year 1 and 37 ml/min/1.73 m^2 at year 5, while seven patients dropped with their eGFR <30 ml/min/1.73 m^2. For-cause biopsies were indicated in one patient in the MSC and eight patients in the control group. In the for-cause biopsy in the MSC group, recurrence of IgA nephropathy was found (n=1). In the control group, acute TCMR IB (n=1), acute TCMR II (n=1), mixed active ABMR and acute TCMR IB (n=1), BK nephropathy (n=2), tubulointerstitial nephritis/pyelonephritis (n=1), IFTA grade III (n=1), and medullary inflammation NOS (sv negative) (n=1) were observed. In the post hoc analyses, none of the seven patients with de novo DSA needed a for-cause biopsy renal biopsy or developed an eGFR <30 ml/min/1.73 m^2. However, it is of importance to note that in three of these seven patients CNI was restarted by their treating nephrologist after the 24-week study period (*Table S4*).

Table 3. *Secondary endpoints (SAE, AE, viral infections, dnDSA) during the study period of 24 weeks.*

Endpoint study period of 24 weeks	MSC group (n=29)	Control group (n=28)
Serious adverse events, total, No.	19	25
Injury, poisoning and procedural complications	6	7
Infections and infestations	2	7
Gastrointestinal disorders	2	3
Renal and urinary disorders	2	2
Metabolism and nutrition disorders	2	2
Therapeutic and nontherapeutic responses	2	1
Investigations	1	1
Vascular disorders	0	1
Musculoskeletal and connective tissue disorders	0	1
Immune system disorders	1	0
Psychiatric disorders	1	0
Adverse events, total, No.	272	301
Investigations	51	46
Blood and lympathic system disorders	39	36
Infections and infestations	32	38
Vascular disorders	35	31
Metabolism and nutrition disorders	26	30
Gastrointestinal disorders	21	32
Renal and urinary disorders	5	17
Injury, poisoning and procedural complications	9	15
General disorders and administration site conditions	10	12
Nervous system disorders	6	10
Musculoskeletal and connective tissue disorders	9	7
Cardiac disorders	10	5
Respiratory, thoracic and mediastinal disorders	5	7
Skin and subcutaneous tissue disorders	8	4
Psychiatric disorders	2	4
Reproductive system and breast disorders	1	2
Neoplasm benign, malignant and unspecified	1	2
Eye disorders	1	2
Immune system disorders	0	1
Ear and labyrinth disorders	1	0
Viral infections, No. (%)		
EBV virus infection[a]	1 (3%)	2 (7%)
CMV virus infection[a]	2 (7%)	3 (11%)
BK virus infection[b]	11 (38%)	10 (36%)
BK nephropathy	1 (3%)	3 (11%)
dnDSA, No. (%)		
Yes	7 (24%)	2 (7%)
Anti-class I	0	0
Anti-class II	4 (14%)	2 (7%)
Anti-class I and II	3 (10%)	0
No	22 (76%)	26 (89%)

MSC = mesenchymal stromal cells, EBV = Epstein-Barr virus, CMV = cytomegalovirus, dnDSA = de novo donor specific antibodies measured at week 24. [a] Peak serum levels (logarithmic) of EBV and CMV range from 2.5 to 3.2 and from 2.7 to 4 respectively. [b] Peak serum levels of BK range from 5.1 to 6.9 in patients with BK nephropathy and from 2.6 to 6.9 in patients without signs of BK nephropathy. [c] dnDSA are considered positive in case of an MFI ≥ 500.

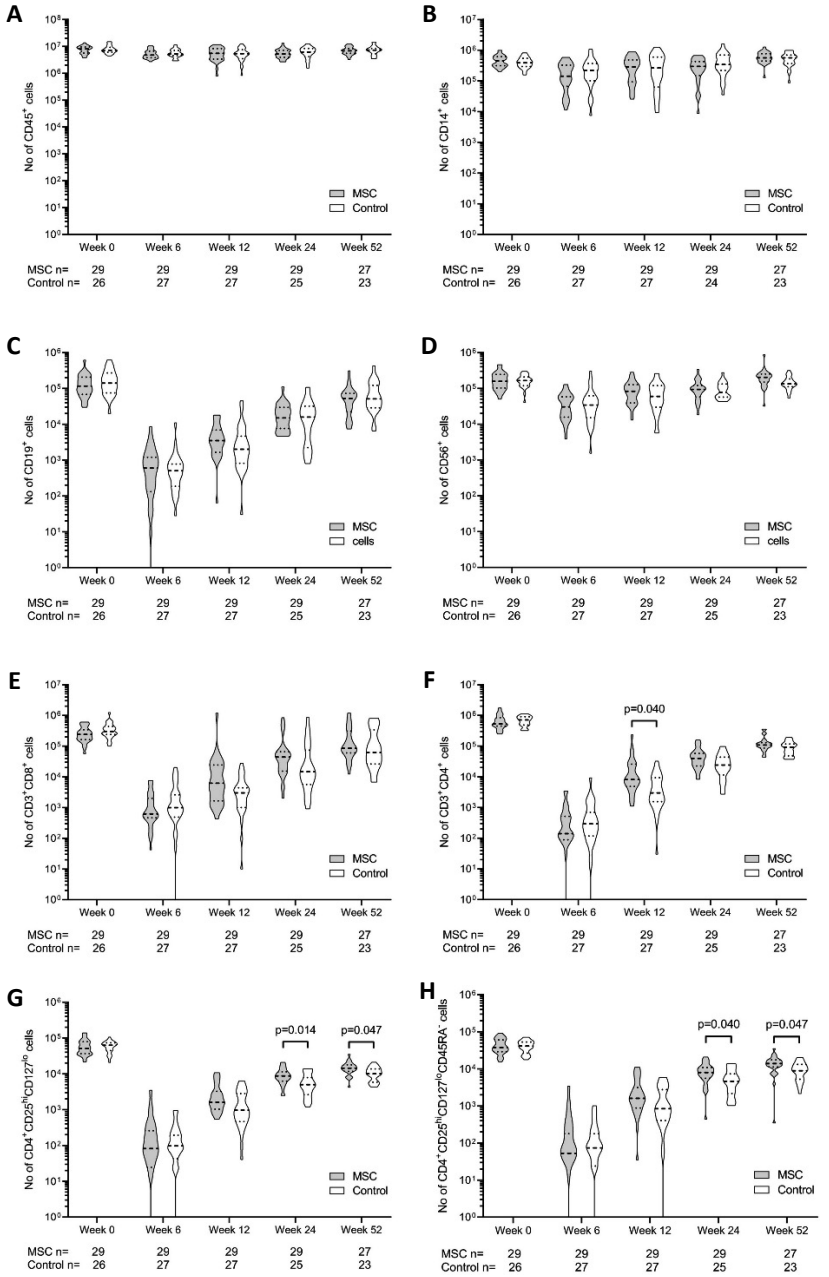

Figure 4. Peripheral blood immune cell composition before and after MSC infusion. *Absolute numbers of (A) CD45⁺ leucocytes, (B) CD14⁺ monocytes, (C) CD19⁺ B cells, (D) CD56⁺ NK cells, (E) CD8⁺ T cells, (F) CD4⁺ T cells, (G) CD4⁺CD25hiCD127lo Tregs, and (H) CD4⁺CD25hiCD127loCD45RA- memory Tregs per mL of blood are shown at baseline before transplantation, before the first MSC infusion (week 6), and time points after both infusions (weeks 12, 24, and 52). Violin plots are given for every time point with the number of individuals studied at each time point below the x-axis. p values are given for the differences between MSC and control groups when <0.05 after Bonferroni correction for multiple testing. MSC = mesenchymal stromal cell; NK = natural killer; Treg = regulatory T cell*

Table 4. *Post-hoc analysis (1-5 years) of endpoints (graft loss, renal function, biopsy scores).*

Endpoint post hoc analysis	MSC group	Control group
1 year	n=26	n=26
2 year	n = 20	n = 20
3 year	n = 10	n = 14
4 year	n = 7	n = 10
5 year	n = 6	n = 7
Graft loss, No.	0	2[a]
Time after Tx, yr		3.8 and 4.5
eGFR, mean (SD) [n], ml/min/1.73m^2		
1 yr	57 (15%) [n=26]	42 (11%) [n=26]
2 yr	55 (15%) [n=20]	39 (12%) [n=20]
3 yr	53 (14%) [n=10]	34 (14%) [n=14]
4 yr	47 (10%) [n=7]	36 (12%) [n=9]
5 yr	50 (20%) [n=6]	37 (15%) [n=5]
eGFR <30ml/min/1.73m2, No.	0	7
Time after Tx, median (IQR), yr		3 (1-3)
Patients with for-cause biopsies, No. (%)	1 (3%)	8 (29%)
Recurrence IgA nephropathy	1	
TCMR IB		1
TCMR II		1
ABMR and TCMR IB		1
BK nephropathy		2
InvestigationsTIN/pyelonephritis		1
IFTA grade III		1
Medullary inflammation		1

MSC = mesenchymal stromal cell, eGFR = estimated glomerular filtration rate, ABMR = antibody mediated rejection, TCMR = T-cell mediated rejection, TIN = tubulointerstitial nephritis, IFTA = interstitial fibrosis and tubular atrophy. All data are described as the total count. Numbers between parenthesis are percentages (also mentioned in the specific variable row).
[a] 1 patient TCMR and recurrence membranous nephropathy; 1 patient chronic transplant dysfunction.

Discussion

In this randomized clinical study, we found that quantitative fibrosis scores and renal function remained stable in patients with MSC therapy and concomitant early tacrolimus withdrawal within the study period of 24 weeks. Only one acute rejection episode was documented in the MSC group after further reduction of clinical immunosuppression in the context of persistent BK viremia/nephropathy. Of interest, there were significantly higher numbers of Tregs in the MSC group with tacrolimus withdrawal compared to the controls. In addition, post hoc analyses demonstrated preserved renal function in the MSC group without evidence of late rejection. Clinical studies with MSCs in kidney transplantation, mainly phase 1 trials with still limited numbers of patients, have demonstrated that MSC treatment after kidney transplantation is safe and feasible.[9-12,20,21] In most studies, MSCs were administered at an early time point against the background of regular immune suppression with the aim to induce immunologic tolerance. The current strategy with MSCs and complete withdrawal of CNI have not been studied before in a randomized trial. Minimization of CNIs is a well-established strategy to limit structural long-term damage to the graft and minimize the side effects associated with clinical immunosuppression.[5,22] A number of trials have demonstrated the efficacy of EVL in conjunction with reduced exposure to CNIs in preventing organ loss or dysfunction in kidney transplant recipients.[23] Of importance, complete avoidance and replacement of a CNI by EVL in de novo transplant recipients are not justified, since unacceptable high acute rejection rates were observed with this strategy.[24] The capability of MSCs to allow reduction of 50% CNI was demonstrated in a previous study with third-party MSCs in 16 living kidney transplant recipients.[21] The combination of an mTOR inhibitor and MSCs was chosen in the current study since experimental evidence demonstrated tolerogenic properties and an increase in regulatory immune cell subsets.[14] In our study, fibrosis scores were similar in both the MSC group and the controls, thereby failing to meet the primary end point, and the incidence of acute rejection 24 weeks after implantation was low. One explanation might be the use of alemtuzumab,[13] which was chosen as we anticipated a higher immunological risk due to the early CNI withdrawal. Indeed, given the potency of the immunosuppression regimen used in our study, seeing differences in fibrosis scores and rejection with the short study duration is unlikely. Of interest, however, the post hoc analysis with follow-up up to 5 years showed a higher incidence of for-cause biopsies in the control group, with findings of both BPAR and BK nephropathy, suggesting that the effect of MSC infusion in combination with CNI withdrawal carried through way beyond the period that alemtuzumab is effective. Future studies with a sufficient number of patients and duration of follow-up are needed to be able to draw more definite conclusions. Several studies have reported an increased incidence of dnDSA in renal transplant recipients receiving EVL, especially when converted early after transplantation, and it was also suggested that the use of alemtuzumab-based induction could aggravate this.[25,26] In general, dnDSA has been shown to be associated with poor graft

survival and increased acute rejection in kidney transplant recipients.[27] In the large ELEVATE Trial, however, conversion to EVL at 10–14 weeks posttransplant was associated with renal function parameters similar to that observed with standard therapy. In this study, the dnDSA data, available in a subset of patients, suggested more frequent anti-HLA Class-I DSA under EVL. Differences in propensity to develop dnDSA, however, did not appear to have resulted in ABMR within the 2-year observation frame of the study.[28] In our study, we also found an increased incidence of dnDSA in patients where tacrolimus was withdrawn. This was associated with (asymptomatic) signs of ABMR in the protocol biopsies of three of these patients of which one, in retrospect, already had subclinical ABMR in the 4-week biopsy. There were no signs of deteriorating graft function in these patients. Furthermore, the post hoc analyses showed no graft losses, no need for additional for-cause biopsies, and stable renal function in these patients as well as the MSC group as a whole. Nevertheless, given the epidemiological association with graft loss (which is, however, based on for-cause DSA measurements), the nephrologists taking care of these patients restarted the CNI in three patients after the study period. Longer follow-up in all patients is warranted to draw more definite conclusions here. Variable outcomes on renal function after MSC therapy have been described and it has been suggested that timing of MSC administration is of major importance. Indeed, early clinical trials have demonstrated an engraftment syndrome with infiltration of immune cells and C3 deposits when MSCs were administered 7 days after renal transplantation, which was not observed when MSCs were given before implantation.[29] In the study by Erpicum et al., eGFR values at day 7 were higher in the MSC-treated patients.[12] In our study, patients in the MSC group started with a higher eGFR, as compared to controls, which was preserved throughout the study period and the post hoc follow-up period. This unequal randomization was, to the best of our knowledge, found by chance and could have influenced our results. In the control group, there was increased graft loss as well as a higher number of patients with inferior renal function (i.e., eGFR <30 ml/min/1.73 m^2), possibly due to an increase in BPAR and BK nephropathy in these patients.

So far, hardly any safety issues have been reported after systemic infusion of MSCs in humans, except for a transient fever and one cardiac event with an unclear causal relationship to the intervention.[12] In our study, there were no side effects directly related to the MSC infusion. We found that (S)AEs (including viral infections) were similar in the two groups. This is in contrast to our previous study where an increased incidence of viral infections was observed after MSC therapy.[10] Possibly this is due to the fact that MSCs were given on top of regular immune suppression in our previous study. This observation is of particular relevance with the ongoing COVID-19 pandemic. Recent observational studies have shown that kidney transplant recipients are at increased risk for severe morbidity due to their systemic immune suppression and often reduced renal function.[30]

MSCs have shown to condition the immune system, by releasing extracellular vesicles or membrane particles or by undergoing apoptosis. This may actively engage recipient monocytes/phagocytes and eventually Tregs, enabling long-term tolerogenic activity that becomes self-sustained even after disappearance of the infused MSCs themselves.[8,31] Of interest, in our current study, we found an increase in the absolute number of Tregs in the MSC group with tacrolimus withdrawal versus control, which has not been reported before in a randomized clinical trial with MSCs in transplant recipients. However, since there was a difference in tacrolimus use between both groups and a difference in total $CD4^+$ T cell counts at week 12, it is not possible to deduce the results solely to the MSC treatment. Concomitantly, the percentage of memory Tregs within total CD4 T cells showed an increase in the control group compared to the MSC group at 12 weeks (*Figure S5*), after which the percentages in total and Treg subsets remained similar, indicating that the increase in absolute Treg numbers in the MSC group is at least partially due to changes in the total $CD4^+$ T cell number.

At present, randomized trials with MSCs are still very limited and the field is only slowly advancing also due to stringent regulatory requirements, the need for clinical grade cell production facilities, and the associated costs. However, we recently also reported the feasibility of administration of third-party "off-the-shelf" MSCs in kidney transplant recipients.[11] This option makes manufacturing and regulation easier and the use of MSC suitable for a wider spectrum of clinical application and much more feasible. We believe that the results of our current trial set the stage for the next steps and use of MSCs in the field of kidney transplantation to reduce the need for excessive use of clinical immunosuppressants.

Acknowledgments

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Supplementary information

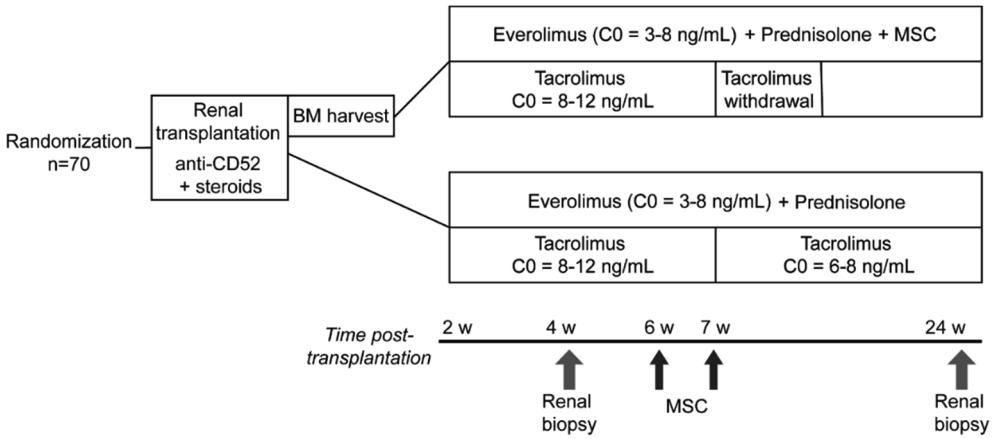


Figure S1. Schematic presentation of study interventions and immunosuppressive regimen. *All patients received prednisolone the entire period. As described in the study protocol, patients received prednisone dose of 100mg (day 1-3), 50mg (day 4), 20mg (day 5-14), 15mg (day 15-21) and 10mg (from day 22). Directly after the 2nd MSC infusion, the MSC group received a higher dose of prednisolone (15mg) for two weeks. In addition patients received alemtuzumab-based induction at day 0 and 1 (15 mg subcutaneously) after transplantation. Target trough level of everolimus was 3-8 ng/mL in both groups. The tacrolimus target was 8-10 ng/mL the first 6 weeks post transplantation and lowered to 6-8 ng/mL in the control group 7 weeks post transplantation. Patients received 2 doses of 1-2.0x10^6 million autologous BM MSC per kg body weight IV, 7 days apart, 6 and 7 weeks after transplantation. Tacrolimus was halved at the time of the second MSC infusion and stopped 1 week later. At that time point patients received 2 weeks 15 mg prednisolone (instead of 10mg).*
MSC = mesenchymal stromal cells, BM = bone marrow, IV = intravenous.

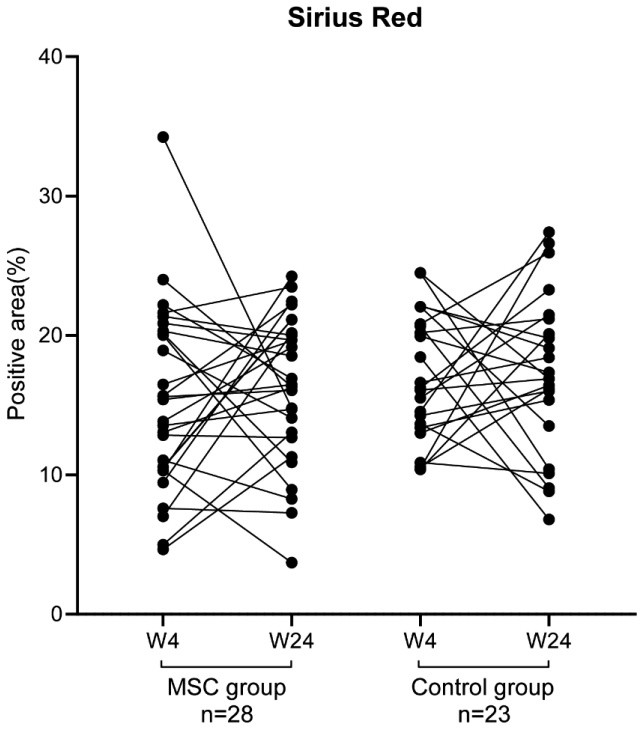
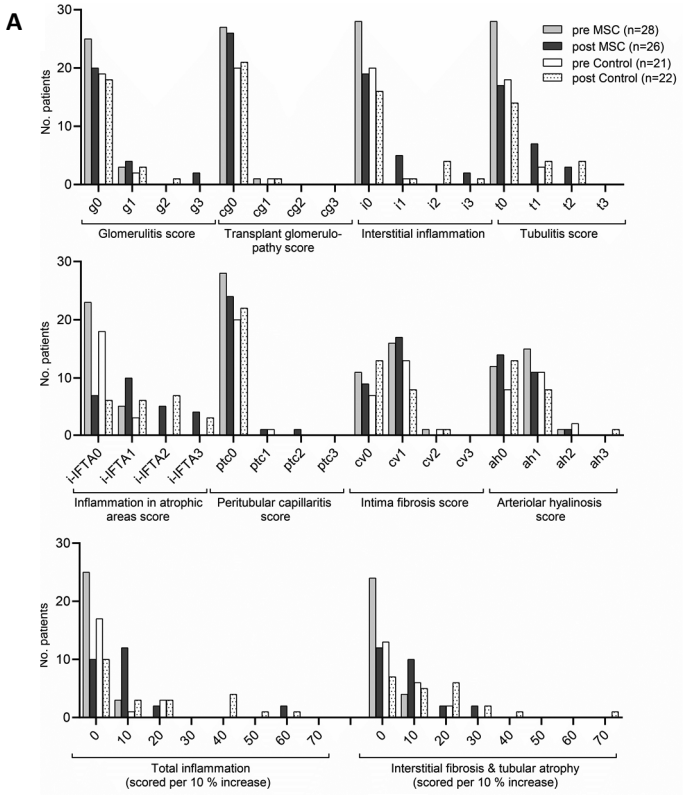


Figure S2. Renal biopsies stained with Sirius Red. *Positive area (Sirius Red Staining) in % in protocol renal biopsies at 4 weeks (W4) and 24 weeks (W24) in the mesenchymal stromal cell (MSC) and control group.*



B

Delta Banff scores between 4 and 24 week protocol biopsy

Characteristic	MSC (n=28)	Control (n=23)	P Value
Glomeruli present – no.	-3 (-32, 25)	1 (-22, 30)	0.2
Globally sclerosed glomeruli - %	0 (-11, 33)	0 (-8, 19)	0.5
g score	0 (-1, 3)	0 (-1, 2)	0.7
cg score	0 (-1, 0)	0 (-1, 1)	0.6
mm score	0 (-1, 0)	0 (-1, 1)	0.2
i score	0 (0, 3)	0 (-1, 2)	0.8
t score	0 (0, 2)	0 (-1, 2)	0.4
ti score	10 (0, 60)	0 (-20, 50)	0.8
IFTA score	0 (-10, 30)	10 (-10, 50)	0.4
iIFTA score	1 (-1, 3)	1 (-1, 3)	0.6
ptc score	0 (0, 2)	0 (-1, 0)	0.1
v score	0 (0, 0)	0 (0, 1)	0.3
cv score	0 (-1, 1)	0 (-1, 2)	0.1
ah score	0 (-1, 1)	0 (-1, 2)	0.4

Data are median (min, max). Statistical test: Wilcoxon rank-sum test

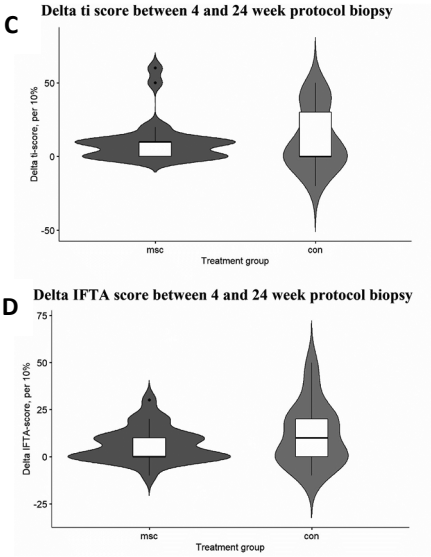


Figure S3. Banff scores of renal biopsies before (4 weeks) and after (24 weeks) transplantation in the mesenchymal stromal cell (MSC) and control group. *Banff scores (from representative biopsies (≥7 glomeruli and 2 vessels)) are depicted as absolute scores (A). Delta Banff scores between 4 and 24 weeks did not differ significantly (B), in particular ti score (C) and IFTA score (D) were comparable.*

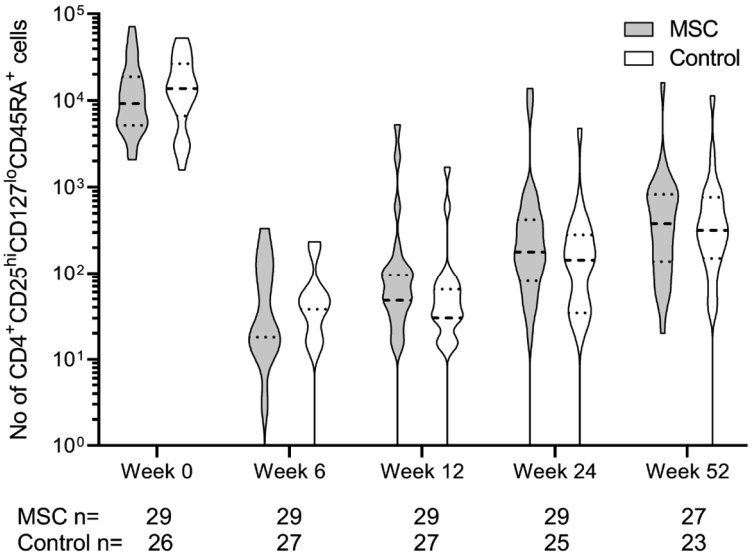


Figure S4. Absolute counts of naive regulatory T cells. *Naive regulatory T cells depicted as absolute counts before transplantation (Week 0) and 6, 12, 24, and 52 weeks after transplantation in the mesenchymal stromal cell (MSC) and control group.*

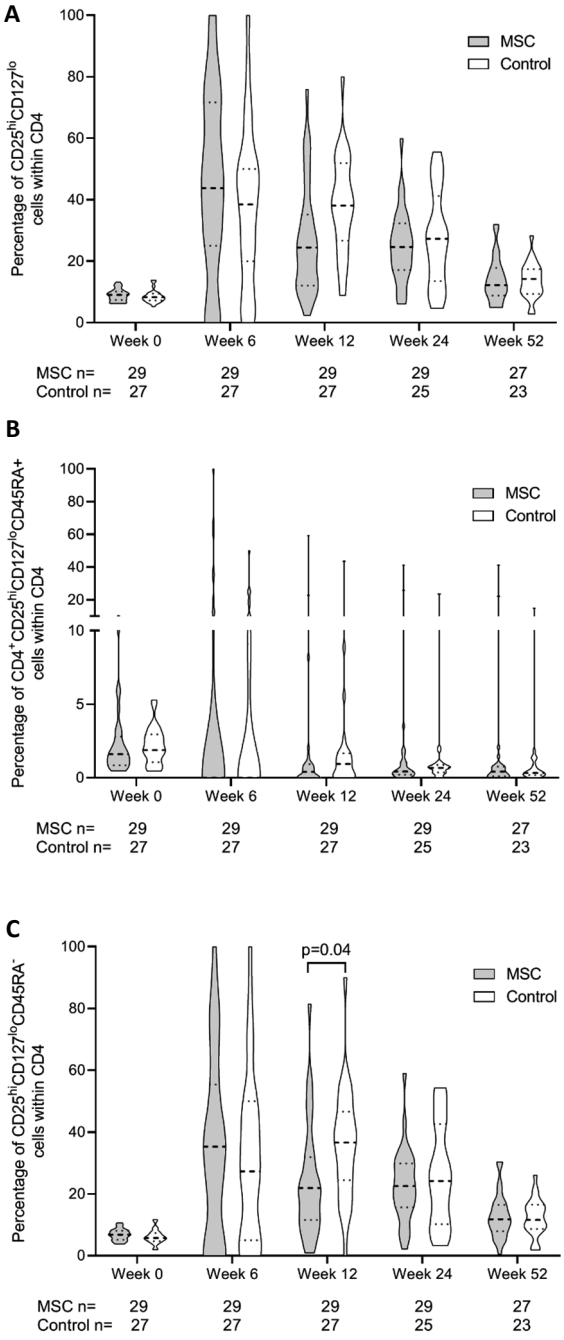


Figure S5. Percentages of total, naïve and memory regulatory T cells within CD4. *Total (A), naïve (B) and memory (C) regulatory T cells depicted as a percentage of the total CD4 count before transplantation (Week 0) and 6, 12, 24, and 52 weeks after transplantation in the mesenchymal stromal cell (MSC) and control group.*

Table S1. *Information and characteristics of processing/expansion of MSC cultures and infusion process.*

Characteristics	n=29
Patient Characteristics	
Age in years, mean (SD)	50 (14)
Male sex, no. (%)	26 (90)
Body weight, kg, mean (SD)	79.4 (14.4)
Culture expansion Characteristics	
Netto volume bone marrow, mL, mean (SD)	119 (11)
MNC in bone marrow ($x10^6$), mean (SD)	440 (273)
Time in culture, days, mean (SD)	22.3 (4.8)
Population doubling level, mean (SD)	3.2 (1.0)
Population doubling time, days, mean (SD)	5.9 (3.6)
Infusion Characteristics	
No. of MSCs administered / kg ($x10^6$), mean (SD)	1.45 (0.13), 1.46 (0.15)
Vitals before administration[a]	143/85, 77, 36.7
Vitals 30 min after administration[a]	141/85, 76, 36.6
Duration 1st (n=29) and 2nd infusion (n=28) mean, min (SD)	21 (12), 20 (10)
Infusion related adverse events	None

From patients allocated to the MSC group, autologous bone marrow mononuclear cells (BM-MNC) were harvested and obtained using ficoll density separation and plated at a density of 160,000 cells per cm^2 in DMEM low-glucose supplemented with 10% fetal bovine serum and antibiotics. At >70% confluency, cells were harvested using TripleSelect, expanded for 1 or 2 subsequent passages to obtain sufficient cells for infusion. After expansion, the final autologous MSC product was frozen and cryopreserved until administration to the patient in 2% human albumin / 0.9% NaCl solution containing 10% DMSO. Characteristics of the MSC infusions are shown. Dosage is given in cells ($x10^6$) per kilogram bodyweight for the first and second infusion. Blood pressure (mm Hg), heart rate (per min) and temperature (^oC) were given as vital signs monitored by MEWS scores (score <2: temp 35-38.5 ^oC, systolic blood pressure 80-200 mm Hg, heart rate 40-110 / minute). As indicated 28 patients received two MSC infusions and 1 patient received one infusion. All data are described as mean and standard deviation (SD). MNC = mononuclear cells, min = minutes. [a] Blood pressure, heart rate, temperature (^oC).

Table S2. *24-hour urine protein measures.*

Visit (weeks)	MSC (n=29)		Control (n=28)		P value[a]
	Total protein (g/24u)	n =	Total protein (g/24u)	n =	
Pre	2·71 ± 2·67	23	1·84 ± 2·19	24	0.12
W4	0·44 ± 0·52	27	0·45 ± 0·37	25	0.35
W6	0·34 ± 0·36	26	0·41 ± 0·28	26	0.14
W7	0·37 ± 0·35	27	0·44 ± 0·29	23	0.20
W8	0·37 ± 0·29	26	0·43 ± 0·35	23	0.30
W9	0·52 ± 0·46	27	0·38 ± 0·27	24	0.38
W10	0·52 ± 0·42	26	0·37 ± 0·33	24	0.11
W12	0·43 ± 0·34	29	0·31 ± 0·2	25	0.23
W14	0·42 ± 0·38	27	0·36 ± 0·26	25	0.87
W16	0·42 ± 0·41	29	0·35 ± 0·2	24	0.72
W20	0·46 ± 0·49	26	0·34 ± 0·2	25	0.89
W24	0·49 ± 0·63	25	0·35 ± 0·32	24	0.42

24 hour proteinuria in mesenchymal stromal cell (MSC) and control group at study visits. All data are described as mean and standard deviation. [#] *24-hour urine protein collected in the year before transplantation.* [a] *Unpaired t test*

Table S4. *Overview of patients on non-protocol immunosuppressive regime at end of study period of 24 weeks.*

	Reason for protocol deviation	n=
MSC group[#]		
Tacrolimus reintroduced	Acute rejection	1
Control group		
Tacrolimus discontinued	BK viremia	2
Everolimus switched to Mycophenolate Mofetil	Thrombo vascular event	4
Everolimus discontinued	CMV infection	1
Everolimus discontinued	Infection of urinoma	1

[#] *In 3 of the 7 patients in the MSC group CNI was reintroduced by their treating nephrologist after the 24 week study period because of dnDSAs developed at week 24.*

Table S3. *Everolimus and Tacrolimus trough blood levels.*

Visit (weeks)	Everolimus (ng/mL)					Tacrolimus (ng/mL)				
	MSC (n=29)		Control (n=28)		P value[a]	MSC (n=29)		Control (n=28)		P value[a]
	Total protein (g/24u)	n =	Total protein (g/24u)	n =		Total protein (g/24u)	n =	Total protein (g/24u)	n =	
W4	5·13 ± 1.5	29	5.47 ± 1.63	26	0.30	8·71 ± 1·97	28	8·51 ± 2·40	28	0·56
W6	5.26 ± 1.27	29	5.13 ± 1.57	23	0.40	7·53 ± 1·93	29	7·23 ± 1·91	27	0·36
W7	5.25 ± 0.9	28	5.34 ± 1.42	23	0.81	7·25 ± 1·50	27	6·44 ± 1·82	25	0·04
W8	5.38 ± 1.51	28	5.75 ± 1.49	24	0.23	3·25 ± 1·00	28	6·42 ± 1·57	26	<0·0001
W10	6.52 ± 1.70	25	5.32 ± 0.97	22	0.01	N.A.		5·38 ± 1·37	26	
W12	6.22 ± 1.64	26	4.90 ± 1.34	22	0.01	N.A.		5·07 ± 1·65	26	
W14	5.87 ± 1.38	26	5.24 ± 1.36	21	0.17	N.A.		5·37 ± 2·12	25	
W16	5.43 ± 1.44	29	5.36 ± 2.31	21	1.00	N.A.		4·85 ± 1·16	25	
W20	5.83 ± 1.35	28	5.17 ± 1.83	19	0.17	N.A.		5·20 ± 1·38	23	
W24	6.18 ± 1.73	28	4.72 ± 1.57	19	0.007	N.A.		4·77 ± 1·66	23	

Trough levels of everolimus and tacrolimus in mesenchymal stromal cell (MSC) and Control group at study visits. Target trough level of everolimus was 3-8 ng/mL. The tacrolimus target was 8-10 ng/mL the first 6 weeks post transplantation and lowered to 6-8 ng/mL in the control group 7 weeks post transplantation. All patients received prednisolone as described in Figure S1. In three patients in the MSC group (10%) and nine patients in the control group (32%) the prednisolone dose was lowered during the 24 week study period to 7.5mg (MSC: none, control: n=3) or 5mg (MSC: n=3, control: n=6) because of a (viral) infection. All data are described as mean and standard deviation. [1] *Unpaired t test*

Chapter 7

Summary and general discussion

Summary

Progression of kidney injury in native and transplanted kidneys has major implications for quality of life and patient survival. Chronic kidney disease led to 1.2 million deaths worldwide and was the 12th leading cause of death in 2017.[1] In addition, CKD led to 35.8 million disability-adjusted life years. Although kidney transplantation improves life quality and life expectancy in most patients, development of injury in kidney grafts leads to severe loss of quality of life in society.[2] Therefore, early recognition and prevention of kidney injury in both native and transplanted kidneys are of vital importance. The current strategy to recognize kidney injury is still dependent upon 'old' biomarkers, such as creatinine and proteinuria, which only recognizes advanced kidney injury. For prevention of kidney injury, physicians currently rely on regulation of blood pressure, minimizing proteinuria and promoting a healthy lifestyle.

Novel biomarkers are needed to recognize kidney injury in an early stage, when serum creatinine or proteinuria lack sensitivity. Therefore, two potential biomarkers, IGFBP7 and TIMP-2, were evaluated in **chapter 2** in the context of progressive kidney injury. These biomarkers proved their added value in the context of acute kidney injury,[3,4] but were not thoroughly investigated in chronic kidney injury. Chapter 2 describes higher circulating levels of both IGFBP7 and TIMP-2 in patients with diabetic nephropathy and to a lesser extend in diabetes patients with a preserved kidney function. IGFBP7 is mainly dependent upon kidney function, while TIMP-2 shows a different picture. As expected, type 1 diabetes patients, who received a simultaneous pancreas-kidney transplantation (SPKT) or kidney transplantation alone (KTA), had lower levels of IGFBP7 levels. However, TIMP-2 did not normalize and persisted to be higher, most likely due to other diabetes-related factors, such as systemic (micro)vascular damage. This finding is supported by a longitudinal study that followed type 1 diabetes patients the first year after SPKT. After one year, lower levels of circulating IGFBP7 persisted, while TIMP-2 levels at one year were comparable with pre-transplant levels. In short, TIMP-2 and IGFBP7 may offer interesting opportunities in monitoring early kidney injury.

Given the extensive amount of vascular injury in diabetes,[5] we next sought to investigate long noncoding RNAs (lncRNAs), since lncRNAs have recently been identified to be associated with vascular injury.[6,7] In **chapter 3**, nine lncRNAs were selected from a panel of 40,173 lncRNAs, in a pilot study of six healthy controls and six patients with diabetic nephropathy. These nine lncRNAs were studied further in the cohort described above. MALAT1, LIPCAR, and LNC-EPHA6 were present at higher circulating levels in patients with diabetic nephropathy. After SPKT MALAT1, LIPCAR, and LNC-EPHA6 normalized within one year. In addition, LIPCAR and LNC-EPHA6 correlated significantly with the vascular marker soluble thrombomodulin, while all three lncRNAs correlated with several vascular specific micro RNAs, supporting the association of these lncRNAs with vascular injury. Taken

together, although additional investigation is warranted, these LncRNAs may provide novel options to monitor vascular injury in diabetes patients.

If progression to end-stage renal disease occurs, kidney transplantation is the preferred treatment, concerning the quality of life and life expectancy.[8] Although transplantation offers several benefits, it comes with uncertainties for the patient. The risk for rejection is always present and injury, as a consequence of rejection, can be severe.[9,10] Since (micro)vascular injury is an important feature of acute rejection,[11] vascular lncRNAs, that we identified in chapter 3, were determined in a cohort of kidney recipients with acute rejection in **chapter 4**. Circulating LNC-EPHA6 appeared to be higher during a rejection episode, compared to healthy controls, and normalized one year after rejection to baseline levels. The correlation between LNC-EPHA6 and soluble thrombomodulin, already described in chapter 3, was confirmed in this cohort. This chapter pointed out the association of LNC-EPHA6 with vascular injury in the context of acute rejection in kidney recipients.

Especially acute antibody-mediated rejection (ABMR) can result in severe injury to the transplanted kidney.[12,13] ABMR is a rare condition and treatment options are only based on little evidence and expert opinion.[14] The primary aim is to avoid ABMR from developing. Risk assessment before transplantation is of vital importance. In **chapter 5**, the incidence and risk factors of ABMR are studied. The vast majority of kidney recipients from an unrelated living donor with ABMR in the first six months after transplantation consists of female recipients, who received a donor kidney from their male spouse. It is suggested that previous pregnancies caused an antibody response in the female recipient against the father of the child (and thus the donor of the kidney). Due to small numbers, a correlation between ABMR and pregnancies in this group was not observed. A retrospective, detailed risk assessment revealed pre-transplant donor specific antibodies (DSAs) in the majority of ABMR patients. The single antigen bead assay identified DSAs in 83% of female recipients of a male spouse, while the current detection strategy only identified 17%. Implementation of the single antigen bead assay as standard work-up in this group may prevent a proportion of ABMR to develop.

Chronic injury in the graft is characterized by the presence of fibrosis in the transplanted kidney and has multiple causes. A major cause of fibrosis formation is the use of calcineurin inhibitors (CNI), as part of the immunosuppressive regime.[15] Therefore, a cellular therapy with mesenchymal stromal cells (MSCs) is described in **chapter 6** with the aim to withdraw CNI at an early time point after renal transplantation. In this randomized controlled trial, MSCs were administered to kidney transplant recipients six and seven weeks after transplantation with subsequent withdrawal of the CNI (tacrolimus). Protocol renal biopsies 4 and 24 weeks after transplantation showed comparable fibrosis between treated patients and a control group with a standard immunosuppressive regime, including a CNI. Withdrawal of tacrolimus did not increase rejection rates significantly (3% in the MSC group)

and slightly less infection related adverse events were documented in the MSC group. Interestingly, regulatory T-cells were significantly higher in MSC patients 24 weeks after transplantation, compared with controls. Therefore, chapter 6 concludes that MSC therapy is a promising alternative for CNI in kidney transplantation, with comparable rejection rates.

General discussion

Early recognition and prevention of kidney injury remains a major challenge. In this thesis, biomarkers, such as IGFBP7, TIMP-2 and specific lncRNAs show their potential as novel means to identify kidney injury. However, before these biomarkers may be implemented in clinical practice, several steps have to be taken. Most importantly, the studies described here did not include patients having an early stage of kidney injury. In addition, prognostic value of these markers for decline in kidney function or kidney failure could not be determined, since this requires follow up of diabetes patients in a very early stage. Nonetheless, they offer the opportunity for further research into these novel biomarkers to identify patients at risk for developing end-stage renal disease in the earliest stage possible.

Chronic kidney injury

In the context of chronic kidney injury, novel biomarkers might be of added value to improve diagnostic approaches and potentially increase knowledge about the pathogenesis. Especially biomarkers in urine and blood can be important tools in the diagnostic process as an easy and cost-efficient way to improve knowledge about the amount of injury in the kidney. In chapter 2, we showed the value of circulating IGFBP7 and TIMP-2 in the discrimination between patients with or without kidney injury or systemic vascular injury, while these already showed their value as urinary biomarkers.[16] Urinary IGFBP7 and TIMP-2 are considered to be related to tubular injury,[17] while our study focusses on IGFBP7 and TIMP-2 in the circulation and their relation with endothelial injury as well. Since DM patients who received an SPKT had higher TIMP-2 levels, the amount of chronic systemic vascular injury, due to the long history of DM, may be associated with circulating TIMP-2 levels. This hypothesis is supported by the correlation with markers of vascular injury and higher levels of TIMP-2 in diabetes patients and chronic injury in kidney transplantation.[18] In chapter 3, we found a similar association between systemic vascular injury in DM patients and three lncRNAs (i.e. MALAT1, LIPCAR, and LNC-EPHA6). In addition, both LNC-EPHA6 and LIPCAR were correlated with the vascular marker soluble thrombomodulin and vascular-injury related micro RNAs. In order to study the early diabetic injury, studying a group of diabetes patients with an early stage of kidney injury, while still having a normal kidney function (eGFR >90ml/min/1.73m^2), would be very interesting. In these patients hyperfiltration occurs and vascular injury is already present. Studying these patients in time would also enable the prediction of development of more severe kidney injury with a decreased eGFR.

In studying noncoding RNAs, such as lncRNAs, it is important to note that noncoding RNAs not only play a role in transcription, splicing, and translation, but do also interact with other types of noncoding RNAs. E.g. Beermann et al. previously described that lncRNAs may function as a sponge for micro RNAs (and thereby alter their expression), next to transcription regulation and posttranscriptional control.[19] Therefore, studying noncoding RNAs should not be limited to one type of noncoding RNA, but should include other noncoding RNAs next to lncRNAs, such as micro RNAs, because of their presumed interactions. In addition, a more robust conclusion can be drawn, because specific micro RNAs are described in vascular injury as well. Most lncRNA levels are expressed at low levels[20], complicating their detection, especially compared to micro RNAs, and this may limit the implementation of lncRNAs in clinical practice. It would be beneficial if more sensitive detection methods would be developed for the clinical use of lncRNAs as biomarkers.

Although clear differences are observed in chapter 2 and 3, the studies described here are not suitable for analysis of the causative relationship between TIMP-2 and lncRNAs with systemic vascular injury. Previous research suggests an active role of both TIMP-2 and lncRNAs in the occurrence of vascular injury.[6,21] TIMP-2 is presumed to alter basement membrane degradation and rebuilding and MALAT1, as an example, is suggested to regulate hyperglycemia-induced endothelial inflammation. Further research is needed to clarify the relationship between these biomarkers and early diabetes related vascular injury. In addition, other factors, such as immunosuppressive drugs, may alter circulating levels of IGFBP7 and TIMP-2, as described before.[22] Nonetheless, we included DM patients with a kidney transplantation alone as a control group for DM patients with a simultaneous pancreas-kidney transplantation, that received largely similar immunosuppressive drugs.

Furthermore, we should be cautious yet in drawing strong conclusions from the studies performed in chapters 2 and 3, due to the limited group size. However, we show interesting changes in diabetes patients and pancreas-kidney recipients, that offer the opportunity to further investigate these groups of biomarkers for the detection and monitoring of chronic vascular injury. Additionally, this may also improve knowledge about the development of diabetes related injury.

Acute rejection

Impairment of kidney function due to vascular injury is not limited to native kidneys. After transplantation, vascular injury is an important cause of renal failure as well. Acute rejection is one of the major causes of vascular inflammation and subsequent injury and graft failure after transplantation. LNC-EPHA6 is described in chapter 3 to associate with vascular injury, due to diabetes and showed the same association with vascular injury due to T-cell mediated rejection in chapter 4. The same trend was observed in LIPCAR levels, without reaching statistical significance. Interestingly, we observed a correlation between LNC-EPHA6 and soluble thrombomodulin in both chapter 3 and 4. Given that sTM is a marker of

endothelial injury and dysfunction,[23] this also suggests a link of LNC-EPHA6 with endothelial cell injury. Previously, lncRNAs AF264622 and AB209021 were described as potential diagnostic biomarkers for acute rejection.[24] Since our study focused on previously selected vascular specific lncRNAs, we did not determine these lncRNAs.

Identification of biomarkers in the context of rejection can be complicated, since rejection frequently coincides with a diminished eGFR with subsequent changes in excretion of the biomarkers. Interestingly, in the longitudinal study, creatinine clearance did not change significantly from 1 month to 12 months after rejection, while we did find changes in vascular injury markers. This emphasizes the importance of sensitive biomarkers to detect vascular damage that doesn't translate into higher creatinine or increased proteinuria. Although speculative, the type of rejection treatment may also affect lncRNA levels. To rule out the influence of rejection treatment, LNC-EPHA6 should be determined in a larger cohort with a standardized rejection treatment. Since ABMR is characterized by even more vascular injury,[14] assessment of LNC-EPHA6 levels in a large ABMR cohort is very interesting. subsequently deteriorated graft function and even graft failure, as we found in chapter 5. A cohort, consisting of kidney recipients with unrelated living donors, was studied to assess the outcome of early acute ABMR in this population. One year graft survival was only 56% in patients with early acute ABMR, compared with 97% in the entire cohort. In the patients with a functioning allograft, kidney function was significantly worse in ABMR patients, compared with recipients with TCMR or no rejection. Interestingly, female recipients from a spousal donor kidney were at risk for ABMR. This is in accordance with previously described cases, where previous pregnancies in particular play a role in the development of preformed donor-specific antibodies and thereby increased risk for ABMR.[25,26] The main limitation in our analysis is that only a small proportion of the cohort developed ABMR. Although ABMR is a rare condition, the severe consequences make prevention of ABMR necessary. Stronger induction therapy (alemtuzumab) does not prevent ABMR in high risk patients. Therefore, more sensitive screening by the single antigen bead assay is needed in high risk populations to lower the initial risk of ABMR.

Chronic injury after kidney transplantation

Minimization of prescription of CNI is one of the strategies to decrease the amount of chronic injury after kidney transplantation.[15,27] However, complete avoidance of CNI leads to unacceptable rejection rates.[28] In chapter 6, a randomized, controlled trial is described, in which kidney recipients receive MSC therapy as a replacement for prescribed calcineurin inhibitors. As previously described, MSCs condition the immune system in different ways, resulting in Tregs that enable self-sustaining tolerogenic activity. In particular in the field of Hematology, MSCs proved their immunomodulatory capacities in graft-versus-host-disease.[29] Interestingly, we found higher numbers of Tregs in the MSC group, compared with the control group. This may have created a favorable immunological state in patients

to withdraw the CNI. After withdrawal of the CNI, also less infectious adverse events were reported in the MSC group.

In both the MSC group and the control group, rejection rates were low. Next to MSC therapy in the MSC group, alemtuzumab induction therapy may also play a role in the low incidence in the MSC patients without CNI. However, long term results (after the therapeutic window of alemtuzumab) show a low incidence of rejection as well. ABMR was not reported in both groups, but patients in the MSC group (and withdrawal of CNI) did have more de novo DSAs. Although these de novo DSAs did not lead to inferior graft survival or graft function, CNI was restarted in these patients.

Unfortunately, still a limited amount of randomized, controlled trials with MSC therapy has been performed worldwide. Further studies are required to increase knowledge about the clinical applications and their potential. We believe, that this study enables the next step to implementation of MSC therapy in the context of kidney transplantation in clinical practice.

In conclusion, we found specific tubular and vascular markers to be associated with the development of chronic kidney injury. Specific vascular lncRNAs increase in diabetic nephropathy and decrease after SPKT. Tubular markers IGFBP7 and TIMP-2 increase in diabetes. IGFBP7 decreases in case of an improved kidney function, while TIMP-2 remains high in patients who received an SPKT. Although these markers are not yet ready for implementation in the diagnostic process, they showed their potential as a biomarker and increase the knowledge about the pathophysiology of development of kidney injury. Secondly, we found the single antigen bead assay to be of added value in the screening process of female kidney recipients from a male spousal donor. This may prevent ABMR after kidney transplantation and therefore improve graft survival. Lastly, we demonstrated MSC therapy to be a feasible alternative for prolonged CNI use in kidney recipients. It was suggested that MSC therapy led to a regulatory response and fibrosis formation did not increase, compared with standard treatment.

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Chapter 8

Addendum

Dutch summary – Nederlandse samenvatting

Curriculum vitae

List of publications

Dankwoord

Dutch summary – Nederlandse samenvatting

Progressie van nierschade in natieve (nieren waarmee iemand wordt geboren) en getransplanteerde nieren heeft grote gevolgen voor de kwaliteit van leven en overleving van de patiënt. Chronische nierziekten leidden in 2017 tot 1,2 miljoen doden wereldwijd en waren de 12e meest voorkomende oorzaak van sterfte.[1] Dit leidde tot 35,8 miljoen verloren levensjaren (gecorrigeerd voor levenskwaliteit). Hoewel niertransplantatie de kwaliteit van leven en levensverwachting van veel patiënten verbetert, beperkt schade in de getransplanteerde nier de gezondheidswinst.[2] Dit maakt vroege herkenning en preventie van nierschade in zowel natieve als getransplanteerde nieren van groot belang. De huidige strategie voor het herkennen van nierschade berust op relatief oude biomarkers als kreatinine en eiwituitscheiding in de urine. Deze markers zijn tekenen van reeds uitgebreide nierschade. Het beperken van progressie van de nierschade wordt momenteel gedaan dor het reguleren van de bloeddruk, verminderen van eiwituitscheiding in de urine en bevorderen van een gezonde levensstijl.

Nieuwe biomarkers zijn van essentieel belang voor het herkennen van nierschade in een vroeg stadium wanneer dit nog niet zichtbaar is met stijging van het serum kreatinine of eiwituitscheiding in de urine. Hiervoor zijn twee potentiële biomarkers, IGFBP7 en TIMP-2, geanalyseerd in **hoofdstuk 2** in de context van progressieve nierschade. De biomarkers bleken eerder reeds van toegevoegde waarde in de context van acute nierschade,[3,4] maar waren nog niet uitgebreid onderzocht in chronische nierschade. Hoofdstuk 2 beschrijft hogere concentraties van IGFBP7 en TIMP-2 in bloed van patiënten met diabetische nefropathie (nierschade ten gevolge van diabetes). In mindere mate is dit tevens het geval bij patiënten met diabetes en een relatief goede nierfunctie. Zoals verwacht waren IGFBP7 concentraties in het bloed van diabetes patiënten die een nier of nier-pancreas transplantatie hadden ondergaan lager. Echter TIMP-2 concentraties bleven hoog in diabetes patiënten na een transplantatie. Dit werd ondersteund door een longitudinale studie waarin diabetespatiënten werden vervolgd in het eerste jaar na een nier-pancreas transplantatie. Na één jaar waren IGFBP7 concentraties persisterend lager, terwijl TIMP-2 concentraties na één jaar weer op vergelijkbaar niveau waren met de waarden van voor de transplantatie. In andere woorden, TIMP-2 en IGFBP7 zijn wellicht interessante biomarkers voor het in kaart brengen van vroege nierschade.

Gezien de uitgebreide vasculaire schade in diabetes patiënten,[5] analyseerden we vervolgens long noncoding RNA's (lncRNAs). Deze lncRNAs zijn blijken bij recent onderzoek te associeren met vasculaire schade.[6,7] In **hoofdstuk 3** zijn in een pilot 40.173 lncRNAs onderzocht in zes gezonde controles en zes patiënten met diabetische nefropathie. Hieruit werden negen veelbelovende lncRNAs geselecteerd en onderzocht in het bovenstaande cohort. MALAT1, LIPCAR en LNC-EPHA6 waren in het bloed in hogere mate aanwezig bij patiënten met diabetische nefropathie. Binnen één jaar na nier-pancreastransplantatie

normaliseerden deze lncRNAs weer. Daarnaast correleerden LIPCAR en LNC-EPHA6 significant met de vasculaire marker soluble thrombomodulin en correleerden MALAT1, LIPCAR en LNC-EPHA6 met enkele vasculair specifieke micro RNA's. Dit ondersteunt de associatie van deze drie lncRNAs met vasculaire schade. In andere woorden, ondanks dat verdere analyse van deze lncRNAs gewenst is, verschaffen deze lncRNAs mogelijk een nieuwe mogelijkheid voor het monitoren van vasculaire schade in diabetes patiënten.

Wanneer sprake is van progressie naar eindstadium nierfalen, is een niertransplantatie de behandeling van voorkeur, wat betreft de levensverwachting en de kwaliteit van leven.[8] Naast de voordelen die een niertransplantatie biedt, gaat een transplantatie ook gepaard met onzekerheden. Er bestaat altijd het risico op het ontstaan van rejectie en de schade die hierop volgt.[9,10] Eén van de karakteristieken van rejectie is (micro)vasculaire schade.[11] Daarom hebben wij de in hoofdstuk 3 geselecteerde lncRNAs onderzocht in een cohort van ontvangers van een transplantatienier met acute rejectie. In **hoofdstuk 4** bleek LNC-EPHA6 verhoogd te zijn tijdens ten tijde van rejectie, vergeleken met gezonde controles. Dit niveau normaliseerde het jaar na transplantatie. De correlatie tussen LNC-EPHA6 en soluble thrombomodulin werd in dit hoofdstuk wederom bevestigd.

Met name antistof gemedieerde rejectie (ABMR) kan leiden tot ernstige schade in een getransplanteerde nier.[12,13] ABMR is een zeldzame aandoening en behandeling is gebaseerd op gebrekkig bewijs en klinische ervaring.[14] Risicoschatting tijdens het opwerken voor transplantatie is van groot belang voor het voorkomen van ABMR na transplantatie. In **hoofdstuk 5** werden de incidentie en risicofactoren van ABMR onderzocht in een groep ontvangers van een nier van een ongerelateerde donor (geen familie). Een evidente meerderheid van de groep met ABMR bleek te bestaan uit vrouwelijke ontvangers die een nier hadden ontvangen van hun echtgenoot. Mogelijk is dit het gevolg van voorgaande zwangerschappen, waarbij in de vrouweijke ontvanger een antistof respons op gang is gekomen tegen de vader van het ongeboren kind (en daarmee tegen de latere donor van de nier). Vanwege kleine aantallen werd een correlatie tussen ABMR en zwangerschappen niet gevonden. Retrospectief bleken donor specifieke antistoffen reeds voor transplantatie aanwezig in de meerderheid van de patiënten met ABMR. Een nauwkeurige methode voor het aantonen van antistoffen (de single bead assay) toonde voor transplantatie donor specifieke antistoffen in 83% van de vrouwelijke ontvangers van een nier van hun echtgenoot. Bij de huidige methode was dit slechts 17%. Kortweg, implementatie van de single antigen bead assay in de standaard risicoschatting voor transplantatie kan bijdragen aan het voorkomen van ABMR in deze groep.

Chronische schade in een transplantatienier wordt gekarakteriseerd door de aanwezigheid van fibrose heeft verschillende oorzaken. Een belangrijke oorzaak van fibrosevorming is het gebruik van een calcineurineremmer als immunosuppressivum.[15] Dit heeft geleid tot het onderzoek in **hoofdstuk 6**, waarbij het doel was gesteld om mesenchymale stromale celtherapie (MSC) te starten als immuunsuppressief alternatief voor een

calcineurineremmer. In een gerandomiseerde, gecontroleerde studie werden MSC's toegediend aan niertransplantatiepatiënten zes en zeven weken na transplantatie, waarna de calcineurineremmer werd gestopt. Protocollaire nierbiopten op 4 en 24 weken toonden vergelijkbare mate van fibrose tussen de controle groep en de groep de MSC groep. Staken van de calcineurineremmer leidde niet tot toegenomen rejectierisico (3% in MSC groep). Interessant om te benoemen is dat regulatoire T-cellen 24 weken na transplantatie significant hoger bleken in de MSC groep vergeleken met de controle groep. MSC therapie lijkt daarom een veelbelovend alternatief voor het gebruik van calcineurineremmers in niertransplantatie met een vergelijkbaar rejectierisico.

Samenvattend vonden we specifieke tubulaire en vasculaire markers die geassocieerd zijn met het ontstaan van chronische nierschade. Specifieke vasculaire lncRNAs nemen toe bij diabetische nefropathie en dalen na nier-pancreastransplantatie. Tubulaire markers IGFBP7 en TIMP-2 zijn toegenomen in diabetes. IGFBP7 daalt wanneer sprake is van een betere nierfunctie, terwijl hoge TIMP-2 levels persisteren na nier-pancreastransplantatie. Ondanks dat deze markers nog niet geïmplementeerd kunnen worden in het diagnostische proces, tonen wij hun potentieel als biomarker en dragen zij bij aan een beter begrip van de pathofysiologie van nierschade. Daarnaast toonden wij de toegevoegde waarde van de single antigen bead assay in de risicoschatting voor transplantatie voor vrouwelijke ontvangers, wat mogelijk gevallen van ABMR kan voorkomen. Tenslotte werd MSC therapie beschreven als een toepasbaar alternatief voor langdurig gebruik van een calcineurineremmer, met vergelijkbare mate van fibrosevorming en een mogelijke regulatoire respons.

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Curriculum vitae

Koen Groeneweg is geboren op 25 maart 1991 te Moerhuizen. Na in 2009 zijn Gymnasium diploma te hebben behaald aan het Oranje Nassau College te Zoetermeer, is hij begonnen aan de opleiding Geneeskunde aan de Universiteit Leiden. In 2012 heeft hij de bachelor Geneeskunde afgerond en in 2015 de master Geneeskunde. Na het afronden van de Geneeskunde studie, heeft hij gedurende één jaar en vijf maanden gewerkt als assistent niet in opleiding bij de Interne Geneeskunde in het Haga Ziekenhuis te Den Haag en het Reinier de Graaf Ziekenhuis te Delft. Aansluitend is hij in juli 2017 gestart met een promotietraject op de afdeling Interne Geneeskunde (Nierziekten) in het Leids Universitair Medisch Centrum, begeleid door Johan W. de Fijter, Marlies E.J. Reinders en Roel Bijkerk. Hierbij werd onderzoek verricht naar nieuwe diagnostische en therapeutische methoden voor het voorkomen van schade in natieve en getransplanteerde nieren. Tijdens het promotietraject was hij voorzitter van het 'Platform AIOS and post-docs Nephrology', nam hij deel aan de organisatie van congressen en was hij de Nederlandse vertegenwoordiger binnen het 'Young Nephrologists' Platform' van de ERA-EDTA. Vanaf 1 januari 2021 is hij begonnen met de opleiding tot internist aan de Erasmus Universiteit te Rotterdam, waarbij het eerste deel van de opleiding zal plaatsvinden in het Reinier de Graaf Ziekenhuis te Delft.

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