Improving renal graft survival

A characterization of antibodies
and complement regulatory proteins

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Improving renal graft survival –
a characterization of antibodies and complement regulatory proteins
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Improving renal graft survival

A characterization of antibodies and complement regulatory proteins

Niertransplantaatoverleving verbeteren –
een karakterisering van antilichamen en complement regulerende eiwitten

(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 17 april 1989
te Tiel

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General introduction and thesis outline

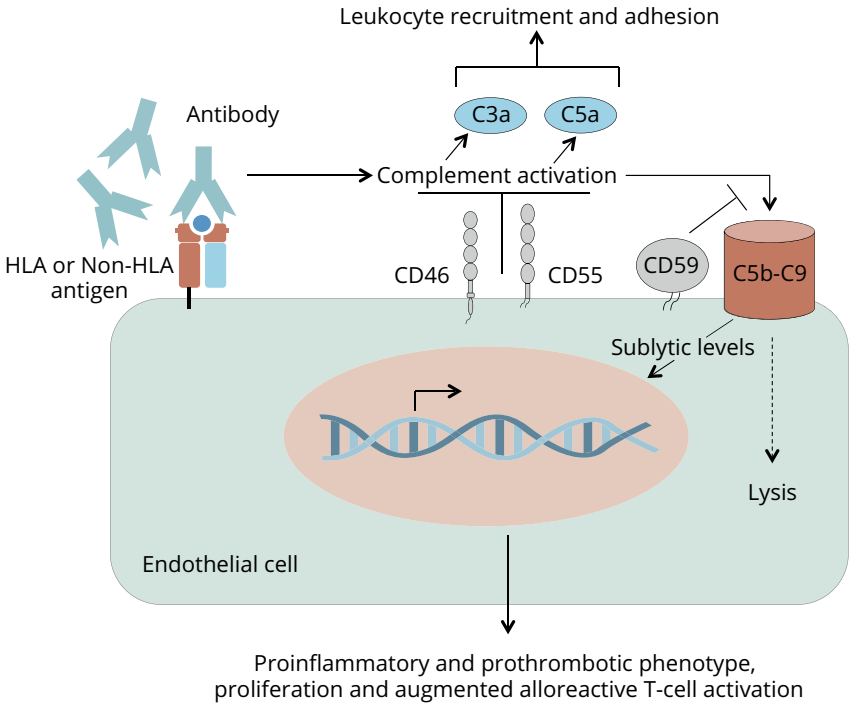
Introduction

A major breakthrough in transplant immunology was the discovery of lymphocytotoxic antibodies directed against the graft in the sera of patients who lost their kidney transplant due to hyperacute rejection [1,2]. Patel and Terasaki subsequently developed a complement-dependent cytotoxicity (CDC) cross-match to test for the presence of these antibodies [2]. In this test, which is still being applied in the clinic, donor lymphocytes are incubated with patient serum and rabbit complement [3,4]. A positive CDC crossmatch, reflected by a substantial degree of lysis of donor lymphocytes, is regarded as an absolute contraindication for transplantation. With the widespread implementation of the CDC crossmatch in clinical practice, hyperacute rejection has almost completely disappeared [5,6]. Nowadays, more sensitive techniques like the flow cytometry crossmatch and solid-phase assays including luminex single antigen bead assays are also available [7-9]. The luminex assay uses magnetic beads coated with HLA antigens instead of donor lymphocytes and therefore can be used for a virtual crossmatch [10]. This very sensitive technique enables additional identification of non-cytotoxic donor-specific anti-HLA antibodies (DSA) and non-donor-specific anti-HLA antibodies [9,10]. Pretransplant non-cytotoxic DSA are associated, at least in deceased donor transplantations, with acute rejection and impaired graft survival [11-15], whereas the significance of non-donor-specific anti-HLA antibodies remains controversial [16-19].

Not all patients with pretransplant DSA develop acute rejection and more than half of these patients still have a functioning graft at 10-years posttransplantation [11-15]. This phenomenon could be due to various aspects including differences in antibody characteristics [20,21], immunosuppressive therapy [22], donor organ quality [23] and effectiveness of the immune response of the recipient [24,25]. Another hypothesis is that some allografts are better capable to protect themselves against antibody-mediated damage than orders. Binding of anti-HLA antibodies to HLA on the donor endothelium can lead to graft damage via antibody-dependent cell-mediated cytotoxicity or complement activation [26-28]. As a protection mechanism against complement activation, donor endothelial cells express complement regulatory proteins (CD46, CD55 and CD59) that prevent further complement activation and formation of the membrane attack complex (C5b-C9), the terminal product of the complement cascade [29]. Membrane attack formation on the donor endothelium may result in cell lysis, while sublytic levels can lead to endothelial cell activation, augment alloreactive T-cell activation and promote a proinflammatory and prothrombotic phenotype (figure 1) [30-33]. Animal studies indicated that accommodated grafts that develop no rejection in the presence of donor-specific antibodies, show upregulation of CD59 on the donor endothelium [34,35]. For CD46 and CD55, human biopsy studies suggested that higher expression levels at time of acute rejection were associated with a favorable graft

function or survival following rejection [36,37]. These observations support the hypothesis that complement regulatory capacities of the donor organ can influence the susceptibility to antibody-mediated complement-dependent graft injury.

Figure 1. Effects of antibody-induced complement activation on the donor endothelium.



Complement split products C3a and C5a induce leukocyte recruitment and adhesion upon binding to their receptors on the donor endothelium (not shown). Sublytic C5b-C9 levels increase transcriptional activity of proinflammatory molecules, growth factors and tissue factor. In CDC-crossmatch negative transplantations, endothelial cell lysis by lytic C5b-C9 (membrane attack complex) levels is rarely observed because of the expression of complement regulatory proteins (CD46, CD55 and CD59) on the donor endothelium. Figure is adapted from [38].

Despite the advancements in HLA antibody detection, antibody-mediated rejection remains the primary cause of graft failure [39]. This illustrates the distinct need for refinement of the current immunologic risk assessment in order to be able to better identify patients at high risk of rejection and graft failure. This evaluation may not only include recipient factors such as HLA antibody status but also novel donor organ characteristics such as complement regulatory capacities. An improved immunological assessment will hopefully aid to a personalized immunosuppressive therapy and follow-up care.

Thesis outline

The overall aim of this thesis was to gain further insights on the role of antibodies and complement regulation in acute rejection and graft survival in kidney transplantation.

Part 1 focuses on the clinical relevance of HLA and non-HLA antibodies in kidney transplantation. In **chapter 2** we studied the impact of pretransplant donor and non-donor-specific HLA antibodies on graft survival in a paired kidney design. Whereas pretransplant DSA are a well-known risk-marker for acute rejection and impaired graft survival, the clinical relevance of non-donor-specific HLA antibodies (nDSA) is much less clear since the available studies show contradictory results. Because these studies were mainly based on relatively small sample sizes, differences between groups can easily occur and affect outcomes. Comparing recipients with a different antibody status but receiving a kidney from the same donor, allows studying the impact of nDSA and DSA on rejection-free and graft survival without the need to account for donor and era-dependent characteristics.

Patients without DSA, receiving a first transplantation are at lower risk of rejection and rejection-mediated graft loss compared with patients who have DSA or have been transplanted before. Immunological low-risk patients may require a different, less intense, immunosuppressive therapy to prevent rejection. Most studies evaluating the effect of initial immunosuppressive therapy on long-term graft survival did not have data on the presence of pretransplant luminex-defined DSA to define immunological low-risk patients. We performed a prospective cohort analysis to assess the effect of initial immunosuppression on long-term kidney transplant outcome in immunological low-risk patients, defined by first transplantation and absence of pretransplant Luminex defined DSA (**chapter 3**).

Patients can form antibodies against targets other than HLA. Multiple targets for these non-HLA antibodies have been identified in kidney transplantation over the last decade. An overview of the identified non-HLA antibodies, their clinical relevance and the mechanisms involved in non-HLA antibody formation is provided in **chapter 4**. Potential triggers that may lead to sensitization against non-HLA antigens are ischemia reperfusion injury and rejection. The induced graft damage may cause expression of intracellular proteins on apoptotic cells, release of donor-derived extracellular vesicles and shedding of extracellular domains of membrane-bound proteins. However, little clinical data on posttransplant sensitization against non-HLA antigens is available to support this hypothesis. Moreover, until recently commercially available non-HLA antibody assays could only test for the presence of a single non-HLA antibody while patients may have non-HLA antibodies against

multiple targets. We used a newly in-house developed non-HLA luminex assay to study pre- and posttransplant sensitization against 12 non-HLA targets and related this to the occurrence of acute rejection (**chapter 5**).

Part 2 focuses on complement regulation in transplantation. Genetic polymorphisms may affect protein levels and function. Multiple polymorphisms in genes encoding for complement (regulatory) proteins have been identified. In **chapter 6** an overview on the studied complement polymorphisms in kidney transplantation and other conditions involving complement activation is provided. In lung transplantation, a polymorphism in the promoter of the gene encoding for CD59 (rs147788946) in donors was associated with incidence of bronchiolitis obliterans syndrome (BOS) following transplantation. We looked in kidney transplantation, whether donor polymorphisms in the promoter of CD59, CD46 and CD55 are also associated with acute rejection and graft survival (**chapter 7**). In **chapter 8** we studied the effect of rs147788946 on gene transcription by using reporter plasmids with the polymorphic promoter sequence.

In **chapter 9** we studied lung transplant recipients instead of kidney transplant patients. CD59 protects cells against complement-mediated damage and may interfere with T-cell activation upon alloantigen binding. It is unknown whether CD59 expression is affected by transplantation. In chapter 9 we compared CD59 expression on different leukocyte subsets pre- and post lung transplantation. A potential mechanism contributing to a lower CD59 expression posttransplantation is shedding. Post lung transplantation, serum soluble CD59 (sCD59) levels are increased in patients with BOS, even before the onset of clinical symptoms. We hypothesized that sCD59 levels in urine and serum are also increased at time of acute rejection in kidney transplantation and may serve as a novel biomarker. Therefore, we measured serum and urine sCD59 levels in serial samples of kidney transplant recipients with and without acute rejection. The results are described in **chapter 10**.

Finally, **chapter 11** provides a general summary of the results described in the previous chapters and discusses these findings to place them in a broader perspective.

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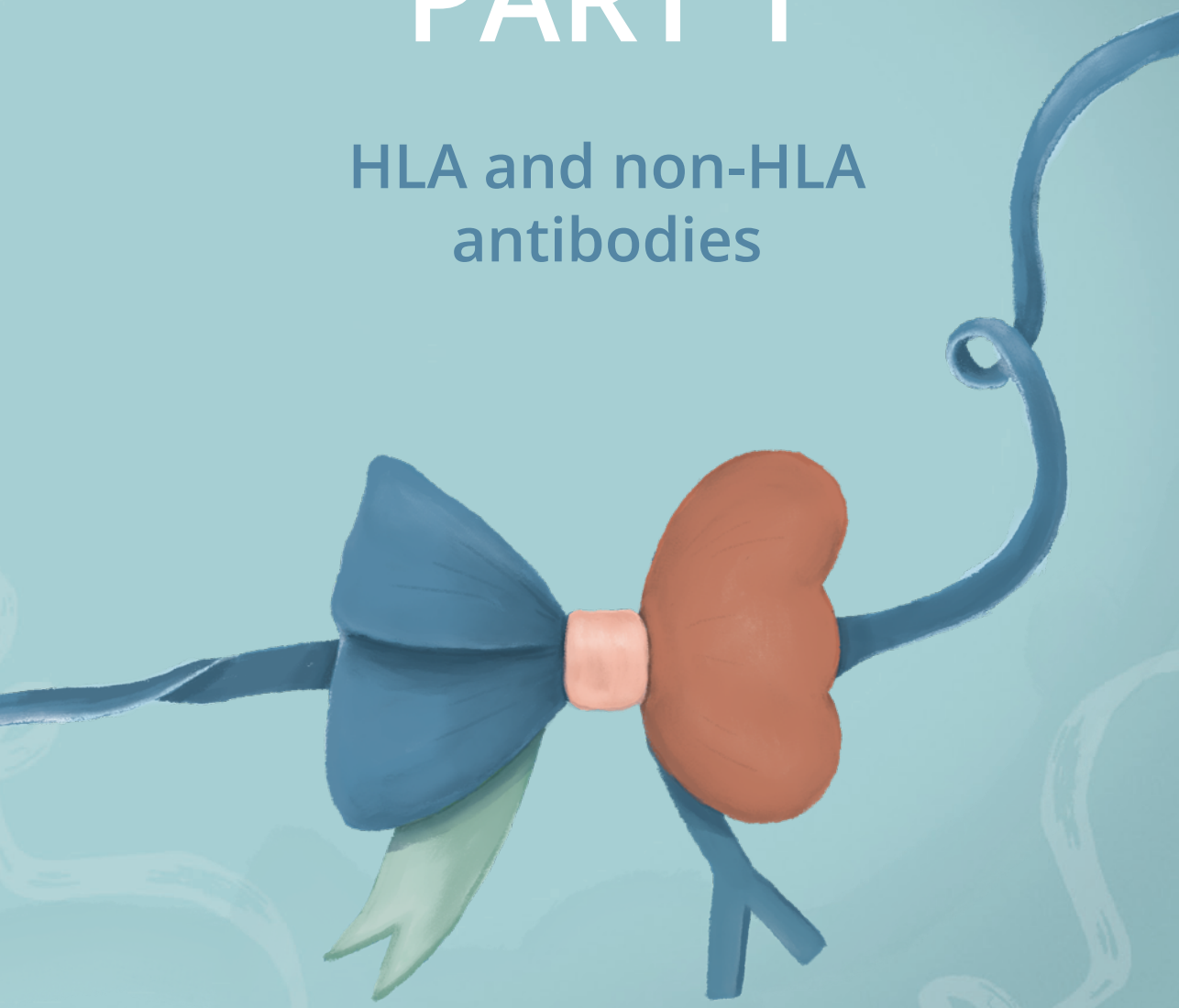
PART 1

HLA and non-HLA antibodies



A paired kidney analysis on the impact of pretransplant anti-HLA antibodies on graft survival

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Nephrol Dial Transplant. 2018; In press.

Abstract

Background

Pretransplant donor-specific anti-HLA antibodies (DSA) are associated with impaired kidney graft survival, while the clinical relevance of non-donor-specific anti-HLA antibodies (nDSA) is more controversial. The aim of the present paired kidney graft study was to compare the clinical relevance of DSA and nDSA.

Methods

To eliminate donor and era-dependent factors, a *post hoc* paired kidney graft analysis was performed as part of a Dutch multicenter study evaluating all transplantations between 1995-2005 with available pretransplant serum samples. Anti-HLA antibodies were detected with a luminex single antigen bead assay.

Results

Among 3237 deceased donor transplantations, we identified 115 recipient pairs receiving a kidney from the same donor with one recipient being DSA positive and the other without anti-HLA antibodies. Patients with pretransplant DSA had a significantly lower 10-year death censored graft survival (55% vs. 82%, p=0.0001). We identified 192 pairs with one recipient nDSA positive (against class I and/or II) and the other without anti-HLA antibodies. For the patients with nDSA against either class I or II, graft survival did not significantly differ compared with patients without anti-HLA antibodies (74% vs. 77%, p=0.79). Only in patients with both nDSA class I and II, there was a trend towards a lower graft survival (58%, p=0.06). Lastly, in a small group of 42 recipient pairs 10-year graft survival in recipients with DSA was 49% compared with 68% in recipients with nDSA (p=0.11).

Conclusion

This paired kidney analysis confirms that the presence of pretransplant DSA in deceased donor transplantations is a risk marker for graft loss, whereas nDSA in general are not associated with a lower graft survival. Subgroup analysis indicated that only in broadly sensitized patients with nDSA against class I and II, nDSA may be a risk marker for graft loss in the long term.

Introduction

Pretransplant donor-specific anti-HLA antibodies (DSA) are a well-recognized and important risk factor for antibody-mediated rejection and graft loss following kidney transplantation [1-4]. DSA binding to HLA-antigens on the donor endothelium may activate a cascade of events including complement activation, resulting in leukocyte recruitment and endothelial cell activation inducing a proinflammatory and prothrombotic phenotype or endothelial cell lysis in the case of lytic membrane attack complex levels [5,6]. Another potential mechanism by which DSA may cause graft injury is antibody-dependent cellular cytotoxicity [5,6]. For non-donor-specific anti-HLA antibodies (nDSA), more controversy on their clinical relevance exists. Some have suggested that nDSA represent an intermediate risk for graft loss and antibody-mediated rejection and require an intensified immunosuppressive strategy [7,8]. The magnitude of the observed impact on 5-year graft survival for patients with nDSA compared with no anti-HLA antibodies in these studies ranged from an absolute difference of 9% to 23%. In contrast, several other studies did not find an inverse association between pretransplant nDSA and graft survival [9-12]. Disparities in donor characteristics and transplant era-dependent factors may interfere with these analyses. Therefore, the aim of this study was to study the impact of DSA and nDSA on kidney transplant outcomes in a paired kidney graft analysis.

Methods

Study design and population

We have performed a *post hoc* analysis on the PROCARE cohort, which includes all deceased and living donor transplantations in the Netherlands between 1995 and 2005 with available pretransplant sera and clinical follow-up [13]. The primary aim of the PROCARE consortium study was to evaluate the impact of pretransplant immunologic factors on long-term kidney graft survival [13]. All transplantations performed during that time required a negative T-cell complement-dependent cytotoxicity (CDC) crossmatch with both peak and current sera. Flow cytometry crossmatching was not routinely performed. Peak and current panel reactive antibodies (PRA) percentage were determined with CDC assays. Solid phase assays for the detection of pretransplant antibodies were not used during that era. Detailed methods on the cohort have been described previously [14]. In brief, pretransplant sera were tested with a Luminex screening assay (Lifecodes LifeScreen, Immucor, Norcross, GA, USA). Positive sera were subsequently tested with a single antigen assay (Lifecodes LSA Class I or Class II kit, Immucor). We used the cut-off as defined by the manufacturer requiring a certain signal to noise ratio to determine bead positivity, which results in virtually identical results as when taking an absolute median fluorescence

intensity (MFI) cut-off of 750. DSA were assigned for HLA-A/B/DR/DQ by comparing bead specificities on serological level with the HLA type of the donor on split level. Because HLA-C and HLA-DP typing was not routinely performed, all anti-HLA-C and -DP antibodies were assigned as nDSA. Clinical data were obtained from the Dutch Organ Transplant Registry. We identified all recipient pairs who received a kidney from the same deceased donor within the PROCARE cohort. Subsequently, we constructed three groups based on a difference in pretransplant anti-HLA antibody status: group 1 (no HLA antibodies versus DSA), group 2 (no HLA antibodies versus nDSA) and group 3 (nDSA versus DSA). Recipient pairs with a similar pretransplant antibody status were excluded from analysis. Recipients and donors were primarily Caucasians. Immunosuppressive treatment was per center discretion and not influenced by pretransplant anti-HLA antibody status as this information was not available at the time of transplantation. Most patients received a combination of a calcineurin inhibitor, an anti-metabolite and prednisolone. Approximately a quarter of the patients received induction therapy with either an Interleukine-2 receptor antagonist or a T-cell depleting agent. Primary outcome was 10-year death-censored graft survival; secondary outcomes were 10-year graft survival uncensored for death and 1-year rejection-free survival. Rejection was defined as receiving treatment for rejection. At the time of the study, biopsies were not performed in all patients receiving treatment for rejection but only on indication. All patients provided written informed consent for use of their clinical data and leftover sera. The study protocol was approved by the Biobank Research Ethics Committee of the UMC Utrecht (TC Bio 13-633) and performed in accordance with the Declaration of Helsinki.

Statistical analysis

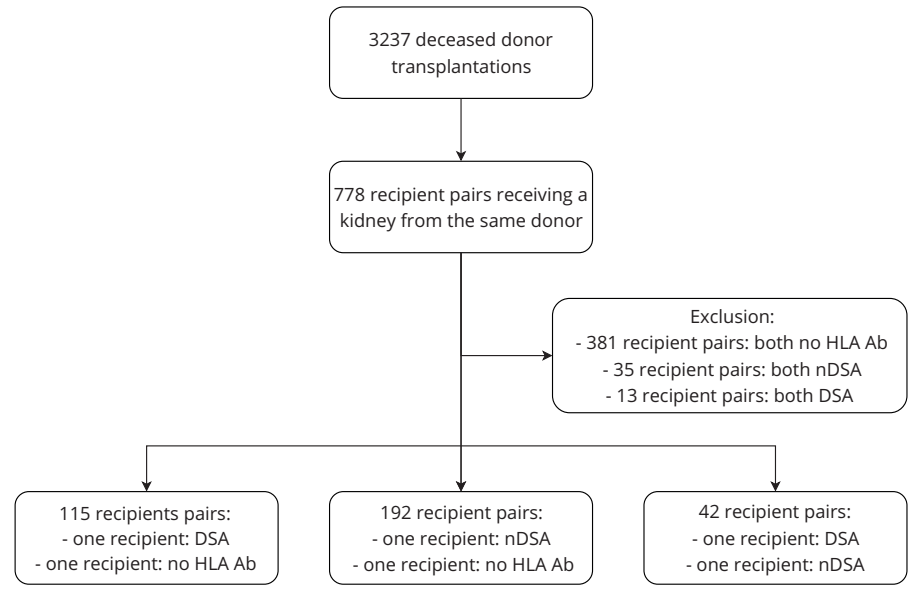
Statistical analyses were done with SAS 9.4 (SAS Institute Inc., Caru, NC, USA) and R 3.2.2. Categorical data were analyzed with the chi-square test and continuous data with the t-test or Mann-Whitney U test. Survival analyses were performed by constructing Kaplan-Meier curves and tested for significance with the log-rank test. With Cox multiple regression, we have adjusted for potential confounders including recipient age, cold ischemia time, induction therapy and calcineurin inhibitor use. To assess the robustness of the results, we additionally performed cumulative incidence analyses and Fine and Gray competing risk regression for 10-year graft and 1-year rejection-free survival [15,16]. For graft survival, death with a functioning graft was considered as a competing event and for rejection-free survival, we considered death with a functioning graft and non-rejection mediated graft loss as competing events. The same covariates as in the Cox multiple regression analyses were entered in the Fine and Gray competing risk regression models.

Results

Baseline characteristics

Between 1995 and 2005, 3237 deceased donor transplantations with available pretransplant sera were performed in the Netherlands. Within this group we identified 778 recipient pairs who received a kidney from the same donor. In the other cases one of the kidneys went to a recipient in another country, one of the kidneys was not suitable for transplantation or pretransplant DSA status was unknown for one of the recipients. In 349 recipient pairs pretransplant anti-HLA antibody status differed (Figure 1). Group 1 consisted of 115 recipient pairs with one recipient being DSA positive and the other recipient without anti-HLA antibodies, group 2: 192 recipient pairs with one recipient with nDSA and the other recipient without anti-HLA antibodies, and group 3: 42 recipient pairs with one recipient with DSA and the other recipient with nDSA, transplanted with a kidney form the same donor.

Figure 1. Flowchart for the in- and exclusion of patients.



Recipient, donor and transplant characteristics for the groups are summarized in Table 1. Compared with their paired recipients, patients with DSA and nDSA in groups 1 and 2 were more often female, retransplants and had a higher median peak PRA. A trend towards a higher usage of induction therapy in patients with DSA in group 1 was observed. Lastly, patients with nDSA in group 2 were treated more frequently with tacrolimus instead of cyclosporine compared with patients without anti-HLA antibodies. Despite these small

Table 1. Baseline characteristics**.**

	Group 1		
	No HLA Ab (n=115)	DSA (n=115)	*p*-value
Donor			
Donor Age	43.7 ± 17.8	43.7 ± 17.8	
Donor Sex, male	67 (58%)	67 (58%)	
Donortype			
• DBD	76 (66%)	76 (66%)	
• DCD	39 (34%)	39 (34%)	
Recipient			
Recipient Age	48.3 ± 13.0	46.6 ± 13.5	0.34
Recipient Sex, male	84 (73%)	50 (44%)	<0.0001
Retransplantation	1 (1%)	53 (46%)	<0.0001
Peak PRA*	0 (0-5)	46 (9-72)	<0.0001
Current PRA*	0 (0-0)	4 (0-34)	<0.0001
Transplantation			
HLA-A, -B, DR mm	2.5 ± 1.0	2.4 ± 1.0	0.32
• HLA-A mm	0.8 ± 0.7	0.8 ± 0.7	
• HLA-B mm	1.0 ± 0.6	1.0 ± 0.6	
• HLA-DR mm	0.7 ± 0.6	0.7 ± 0.5	
CIT (hours)	20.7 ± 7.8	21.7 ± 6.3	0.12
Induction			0.07
• Il2rMoAb	24 (21%)	27 (24%)	
• T-cell depleting	2 (2%)	9 (8%)	
CNI			0.11
• Cyclosporine	61 (53%)	60 (61%)	
• Tacrolimus	44 (38%)	42 (37%)	
• None	10 (9%)	3 (3%)	
Antimetabolite			0.72
• MMF/MPA	86 (75%)	91 (79%)	
• Azathioprine	2 (2%)	2 (2%)	
• None	27 (23%)	22 (19%)	
Steroids	115 (100%)	113 (98%)	0.16

Data are depicted as number (percentage) or mean ± standard deviation unless otherwise stated.

* Median (interquartile range).

	Group 2			Group 3	
No HLA Ab (n=192)	nDSA (n=192)	*p*-value	nDSA (n=42)	DSA (n=42)	*p*-value
41.6 ± 16.5	41.6 ± 16.5		45.7 ± 14.9	45.7 ± 14.9	
108 (56%)	108 (56%)		24 (57%)	24 (57%)	
119 (62%)	119 (62%)		29 (69%)	29 (69%)	
73 (38%)	73 (38%)		13 (31%)	13 (31%)	
47.6 ± 13.0	50.4 ± 13.0	0.05	48.2 ± 13.0	46.4 ± 12.5	0.48
142 (74%)	89 (46%)	<0.0001	16 (38%)	18 (43%)	0.65
7 (4%)	64 (33%)	<0.0001	15 (36%)	23 (55%)	0.08
0 (0-4)	8 (0-44)	<0.0001	18 (3-55)	66 (37-85)	0.0009
0 (0-0)	0 (0-3)	<0.0001	0 (0-6)	32 (0-55)	0.002
2.3 ± 1.1	2.3 ± 1.0	0.77	2.1 ± 1.3	2.1 ± 1.1	0.93
0.8 ± 0.6	0.8 ± 0.6		0.6 ± 0.6	0.7 ± 0.6	
1.0 ± 0.6	0.9 ± 0.6		1.0 ± 0.6	1.0 ± 0.7	
0.7 ± 0.6	0.7 ± 0.6		0.5 ± 0.6	0.4 ± 0.5	
21.2 ± 7.3	21.9 ± 6.7	0.37	22.9 ± 7.5	24.5 ± 7.0	0.41
		0.31			0.45
49 (26%)	41 (22%)		8 (19%)	6 (14%)	
3 (2%)	7 (4%)		2 (5%)	5 (12%)	
		0.005			0.45
113 (59%)	90 (47%)		27 (64%)	24 (57%)	
65 (34%)	95 (49%)		12 (29%)	17 (41%)	
14 (7%)	7 (4%)		3 (7%)	1 (2%)	
		0.88			0.19
139 (72%)	137 (71%)		29 (69%)	33 (79%)	
7 (4%)	9 (5%)		3 (7%)	0	
46 (24%)	46 (24%)		10 (24%)	9 (21%)	
188 (98%)	188 (98%)	1	42 (100%)	41 (98%)	0.31

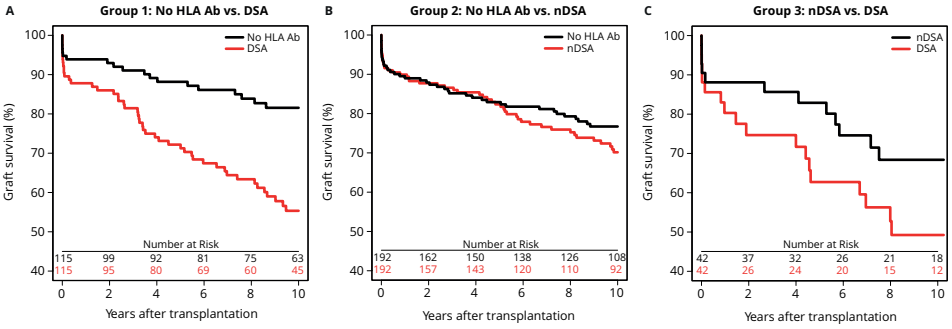
Abbreviations: Ab, antibodies; CIT, cold ischemic time; CNI, calcineurin inhibitor; DBD, donation after brain death; DCD, donation after circulatory death; DSA, donor-specific anti-HLA antibodies; Il2rMoAb, Interleukin 2 receptor monoclonal antibody; mm, mismatch; MMF, mycophenolic mofetil; MPA, mycophenolic acid; nDSA, non-donor-specific anti-HLA antibodies; PRA, panel reactive antibodies.

differences, overall immunosuppressive treatment among the different group was largely comparable. Peak PRA was higher in the nDSA patients in group 3 compared with the nDSA patients in group 2. The mean number of HLA-A/-B/-DR mismatches seemed slightly lower in group 3 (2.1 ± 1.2) compared with groups 1 and 2 (2.3 ± 1.0), no significant differences within groups were observed. For the patients with DSA, the median cumulative MFI of DSA against class I and II was 4351 (IQR 1634-12021) in group 1 and 5084 (IQR 1997-12527) in group 3.

Pretransplant anti-HLA antibody status and graft survival

Ten-year death-censored graft survival was significantly lower in patients with DSA compared with the paired recipients without anti-HLA antibodies (55% versus 82%, figure 2A). Patients with DSA against both Class I and II had the lowest graft survival (n=22, 43%), while the survival rate was 57% and 59% for patients with either Class I or Class II DSA (n=58 and n=35, overall *p*=0.0006, supplementary figure 1). When combining graft failure and death with a functioning transplant, graft survival remained lower for patients with DSA (44%) compared with the paired patients without anti-HLA antibodies (57%, *p*=0.046). After adjustment for recipient age, cold ischemia time and induction therapy, DSA was still associated with a lower death-censored and uncensored graft survival (table 2).

Figure 2. Death-censored 10-year graft survival stratified according to pretransplant anti-HLA antibody status.

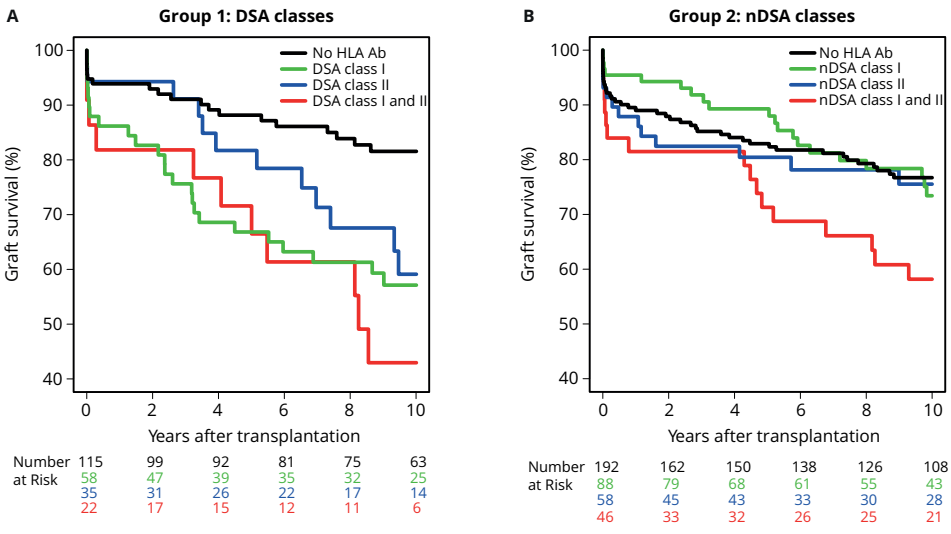


(A) DSA compared with no anti-HLA Ab (55% vs. 82%, *p*=0.0001). (B) nDSA compared with no anti-HLA Ab (70% vs. 77%, *p*=0.27). (C) DSA compared with nDSA (49% vs. 68%, *p*=0.11).

Overall 10-year death-censored graft survival did not differ significantly between recipient pairs with nDSA and without anti-HLA antibodies (*p*=0.27). However, as shown in figure 2B the curves are almost identical during the first 5 years, whereupon they seem to diverge slightly in disadvantage of the patients with nDSA. To investigate this further, we stratified patients with nDSA based on HLA classes. Patients with nDSA Class I and II showed a markedly higher peak PRA (median 36, IQR 11-53) and more often had a retransplant (61%) as compared with patients with nDSA against either Class I or II (supplementary

table 1). Death-censored graft survival was similar for patients with nDSA Class I (73%) or Class II (76%) as compared with patients without anti-HLA antibodies throughout the entire follow-up period, indicating no association between nDSA against either Class I or II and long-term graft survival (figure 3B). Whereas, the graft survival curve suggests a lower graft survival in patients with nDSA Class I and II (58%, overall p=0.11, figure 3B). Compared with their paired recipients without anti-HLA antibodies, patients with nDSA Class I and II tended to have a lower 10-year death-censored graft survival (p=0.06). Grouping patients with nDSA in tertiles based on their maximum or cumulative MFI of the positive beads did not identify any significant correlation with death-censored graft survival (p=0.21 and p=0.66). Death with a functioning graft occurred slightly more often in the nDSA group, resulting in a lower overall graft survival of 50% compared with 63% in the group without anti-HLA antibodies (p=0.02). After adjustment for potential confounders including recipient age, which was on average slightly higher in the nDSA group, this difference was not significant anymore (HR 1.28, 95% CI 0.93-1.76, table 2).

Figure 3. Death-censored 10-year graft survival stratified according to DSA and nDSA classes.



(A) Graft survival was lower in patients with DSA class I and II (43%) compared with patients with either class I or II DSA (57%, 59%) or no HLA antibodies (82%, overall p=0.0006). (B) Graft survival was lower in patients with nDSA class I and II (58%) compared with patients with either class I or II nDSA or no HLA antibodies (overall p=0.11).

Finally, we compared death-censored graft survival among 42 recipient pairs with one being DSA positive and the other nDSA positive (figure 1C). Within this small group, we observed a trend towards a lower death-censored graft survival in patients with DSA (49%) as to patients with nDSA (68%, p=0.11) and a similar trend for overall graft survival (p=0.05).

One-year rejection-free survival censored for death and failure unrelated to rejection did only differ between recipients with DSA (61%) compared with patients without anti-HLA antibodies (78%, adjusted HR 2.15, 95%CI 1.29-3.56), but not between the other groups (table 2).

Table 2. Transplant outcomes compared among groups of recipients with different anti-HLA antibody status.

	DSA vs. no HLA Ab	**nDSA vs. no HLA Ab**	**DSA vs. nDSA***
	HR (95% CI)	HR (95% CI)	HR (95% CI)
10-year death-censored graft failure	2.75 (1.61-4.68)[1]	1.26 (0.84-1.90)[1]	1.81 (0.87-3.76)[1]
	2.94 (1.69-5.10)[2]	1.10 (0.73-1.68)[2]	-
10-year graft failure including death with a functioning graft	1.46 (1.01-2.12)[1]	1.46 (1.07-1.99)[1]	1.74 (0.99-3.06)[1]
	1.56 (1.06-2.28)[2]	1.28 (0.93-1.76)[2]	-
Rejection within year 1	2.12 (1.29-3.50)[1]	0.79 (0.53-1.17)[1]	1.14 (0.54-2.42)[1]
	2.15 (1.29-3.56)[2]	0.82 (0.55-1.23)[2]	-

[1] Crude analysis.

[2]Adjusted analysis (recipient age, cold ischemic time, calcineurin inhibitor and induction therapy).

*No adjusted analyses were performed for this group because of the limited number of patients.

Abbreviations: CI, confidence interval; HR, hazard ratio.

Competing risk analysis

To test the robustness of our findings we also performed a competing risk analysis to rule out any major hinder of death as a competing event on the association between pretransplant antibody status and graft survival. Cumulative incidence curves were in line with the complement of the Kaplan-Meier curves (supplementary figure 1). The sub-distribution hazard ratios were also within the range of the hazard ratios that were obtained by Cox regression (supplementary table 2). For 1-year rejection-free survival we also performed a competing risk analysis, with death with a functioning graft and failure unrelated to rejection as competing events. As with graft survival, the cumulative incidences and sub-distribution hazard ratios were in line with the hazard ratios that were obtained by Cox regression (supplementary table 2).

Discussion

The results of this paired kidney graft analysis confirm the association between pretransplant DSA and incidence of rejection and graft loss. For nDSA however, we did not observe such an adverse association with transplant outcomes. Subgroup analysis revealed that only the combination of nDSA against Class I and II were associated with a lower death-censored graft survival in the long term, although this did not reach statistical significance.

A major strength of this study is the paired kidney design. Multivariable analyses have a limited accuracy to adjust for differences in donor organ quality and other era-dependent changes, partly due to the presence of unmeasured confounders [17]. By performing a paired kidney analysis we have eliminated these factors. Within the remainder of the PROCARE cohort graft failure, including death with a functioning graft, did not differ between patients with nDSA and without anti-HLA antibodies (p=0.33, unpublished data). In our paired analysis, the incidence of death with a functioning graft was slightly higher in patients with nDSA. This could be the result of a higher recipient age in the nDSA group. Indeed, we did not observe a significant difference anymore after adjusting among others for recipient age.

Most studies evaluating the effect of pretransplant DSA on transplant outcomes compare DSA positive and DSA negative patients without making a distinction between patients without anti-HLA antibodies and patients with nDSA [3,18,19]. The few studies that did make this distinction had relatively small sample sized and reported conflicting outcomes on the clinical relevance of Luminex-defined nDSA. Several studies did not find a difference in graft survival [9-12]. In contrast, Richter et al. showed in a population of 197 transplant patients that the presence of nDSA (n=39) was associated with a markedly lower 5-year death censored graft survival (62.2% versus 90.8%, HR 4.91, 95%CI 1.43-16.91) [7]. Malheiro et al. reported in a cohort consisting of 756 transplantations a smaller, but significant difference in 5-year graft survival between patients with nDSA and patients without anti-HLA antibodies (88% versus 94%, HR 2.24, 95%CI 1.19-4.37) [8]. Importantly, in both studies the average number of HLA-A, -B and -DR mismatches was higher than in our population [7,8]. Richter et al. showed in immunized patients (nDSA and/or DSA) that graft survival was primarily affected in patients with HLA-DR mismatches [7]. Because of our relatively well-matched population, we cannot exclude an effect of nDSA on graft survival in the presence of multiple HLA-DR and linked -DQ mismatches [20].

One of the hypothesized mechanisms by which the presence of nDSA is associated with impaired graft survival is that they reflect the potential to form HLA antibodies upon allorecognition [7,8]. This could indicate that these patients may be more prone to develop *de novo* DSA [20]. However, there is limited evidence actually supporting a correlation

between pretransplant nDSA and *de novo* DSA [21]. These patients could also have previously formed donor-specific antibodies that are not detectable at time of transplantation but that can become rapidly detectable posttransplantation because of resident HLA-specific memory B-cells [20]. HLA-specific B-cell ELISPOT assays could provide additional insights on this [22,23]. Another suggested mechanism is that nDSA can contribute to graft injury by epitope sharing between the nDSA specificities and donor-specific antigens [24,25]. The suggested reduced graft survival in patients with nDSA against Class I and II, might rather be the result of the high number of retransplants and subsequent broad allosensitization as reflected by a median peak PRA of 36 (IQR 11-53). Peak PRA levels have been associated with graft survival in several studies [26-28]. The chance of epitope sharing between nDSA specificities and donor-specific antigens might also be greater in these more broadly sensitized patients.

This study also has some limitations. We did not have information on the development of *de novo* DSA following transplantation, a well known risk indicator for rejection and poorer graft survival [29], as routine DSA monitoring posttransplantation was not performed at the time of transplantation and follow-up of this cohort. Furthermore, we assigned all anti-HLA-C and -DP antibodies as nDSA since HLA-C and -DP typing was not routinely performed. Because of the low prevalence of isolated pretransplant anti-HLA antibodies against HLA-C or -DP it is difficult to determine their exact clinical relevance [30,31]. Several lines of data, primarily case-reports and case-control studies in sensitized patients with isolated DSA against HLA-C or -DP, suggest that these antibodies are also associated with acute rejection and graft loss [32-34]. After exclusion of patients with nDSA against HLA-C or -DP form group 2, the graft survival lines came even closer together (supplementary figure 2). We cannot rule out that a few patients with nDSA against HLA-C or -DP were misclassified. Though, we still observed a similar trend towards a lower graft survival in patients with nDSA against Class I and II compared with patients with no antibodies and no difference in graft survival for patients with nDSA against Class I or II, indicating no severe bias of any potential misclassification. Another limitation of this retrospective study is that we had only limited data on rejection and could not distinguish between antibody- or cellular-mediated rejection. Pretransplant DSA are typically associated with antibody-mediated rejection [9] and it has been suggested that graft survival in patients with pretransplant DSA is primarily affected in patients who develop AMR within the first year [35]. However, even if biopsy data would have been available, it is likely that some of the earlier biopsies would have been classified different later on as the Banff classification underwent major evolvement over time of this cohort [36]. Finally, the results of our analysis may not be extendable to all patient groups as the cohort compromises primarily Caucasian recipients and donors, is relatively well HLA-matched and patients are in general at immunological low-risk as indicated by the peak and current PRA.

In conclusion, this paired kidney analysis confirms the adverse association between pretransplant DSA and graft survival, suggesting that pretransplant DSA status should be considered in deceased donor transplantations in terms of donor acceptance and immunosuppressive treatment. Whereas, pretransplant nDSA per se are not an additional risk marker for graft failure in immunological low-risk deceased donor transplantations. Only in broadly sensitized patients with nDSA against Class I and II, nDSA may be a marker for graft loss on the long term. These results could indicate that there is no need for intensified immunosuppressive therapy like T-cell depleting agents or increased dosages of maintenance drugs in patients with pretransplant nDSA against either class I or II and no other distinct immunologic risk makers.

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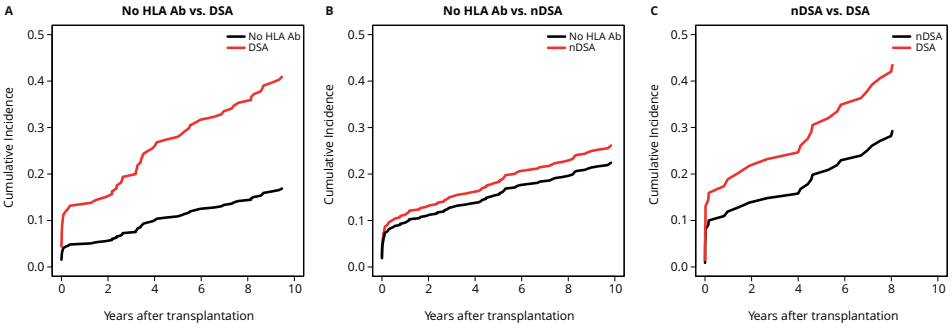
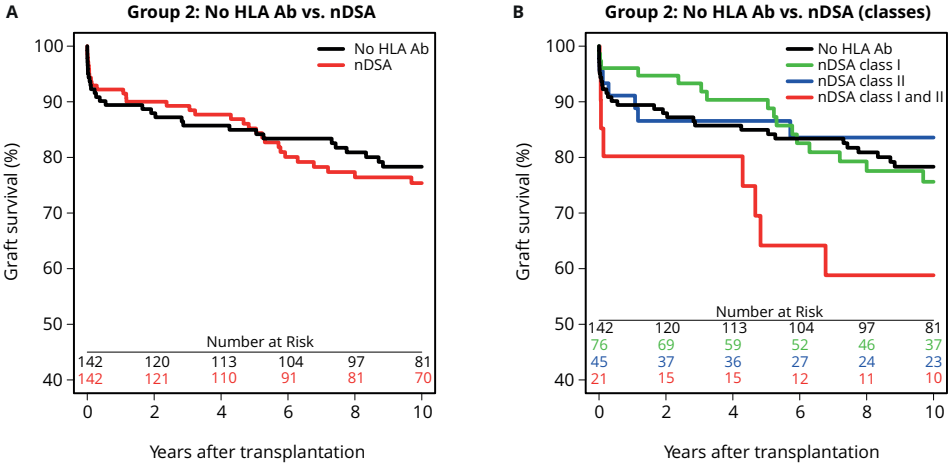
Supplementary material

Supplementary figure 1. Cumulative incidence of graft failure stratified according to pretransplant anti-HLA antibody status.



Death with a functioning transplant was considered as a competing event. (A) DSA compared with no anti-HLA Ab (0.17 vs. 0.42, *p*=0.0001). (B) nDSA compared with no anti-HLA Ab (0.26 vs. 0.22, *p*=0.40). (C) DSA compared with nDSA (0.44 vs. 0.29, *p*=0.18).

Supplementary figure 2. Death-censored 10-year graft survival for patients with nDSA compared with their paired recipients with no anti-HLA Ab, excluding patients with anti-HLA antibodies against HLA-C and/or -DP.



(A) nDSA compared with no anti-HLA Ab (75% vs. 78%, *p*=0.67). (B) Graft survival in patients with nDSA class I and II (59%) was lower compared with patients with either class I or II nDSA or no HLA antibodies (overall *p*=0.18).

Supplementary table 1. Baseline characteristics stratified according to nDSA classes for group 2.

	No HLA-antibodies	nDSA class I	nDSA class II	nDSA class I and II	*P*-value
Number	190	88	58	46	
Donor					
Donor Age	41.6 ± 16.5	42.4 ± 16.9	39.2 ± 17.6	42.9 ± 14.2	0.47
Donor Sex, male	108 (56%)	45 (51%)	36 (62%)	27 (59%)	0.40
Donortype					
• DBD	119 (62%)	56 (64%)	36 (62%)	27 (59%)	0.86
• DCD	73 (38%)	32 (36%)	22 (38%)	19 (41%)	
Recipient					
Recipient Age	47.6 ± 13.0	52.2 ± 12.4	49.5 ± 13.6	48.2 ± 13.2	0.20
Recipient Sex, male	142 (74%)	36 (41%)	32 (55%)	21 (46%)	0.24
Retransplantation	7 (4%)	19 (22%)	17 (29%)	28 (61%)	<0.0001
Peak PRA*	0 (0-4)	14 (0-51)	0 (0-8)	36 (11-53)	<0.0001
Current PRA*	0 (0-0)	0 (0-3)	0 (0-2)	1 (0-15)	0.01
Transplantation					
HLA-A, -B, DR mm	2.5 ± 1.2	2.4 ± 1.2	2.5 ± 1.1	2.2 ± 1.1	0.58
CIT (hours)	21.2 ± 7.3	21.0 ± 6.6	22.9 ± 7.4	22.4 ± 5.8	0.14
Induction					0.12
• Il2rMoAb	49 (26%)	24 (27%)	7 (12%)	10 (22%)	
• T-cell depleting	3 (2%)	5 (6%)	1 (2%)	1 (2%)	

p-values are obtained from an overall comparison amongst the different nDSA groups.

* Median (interquartile range).

Abbreviations: CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after circulatory death; Il2rMoAb, Interleukin 2 receptor monoclonal antibody; mm, mismatch; PRA, panel reactive antibodies.

Supplementary table 2. Sub-distribution hazard ratios (Fine & Gray regression) for graft failure and rejection.

	DSA vs. no HLA Ab	nDSA vs. no HLA Ab	DSA vs nDSA*
	sHR (95% CI)	sHR (95% CI)	sHR (95% CI)
10-year graft failure	2.85 (1.67-4.87)[1]	1.19 (0.79-1.79)[1]	1.65 (0.80-3.39)[1]
	3.05 (1.70-5.48)[2]	1.05 (0.68-1.63)[2]	-
Rejection within year 1	2.12 (1.31-3.45)[1]	0.79 (0.53-1.17)[1]	1.11 (0.53-2.34)[1]
	2.19 (1.34-3.58)[2]	0.84 (0.56-1.26)[2]	-

[1] Crude analysis.

[2] Adjusted analysis: recipient age, cold ischemic time, calcineurin inhibitor and induction therapy.

* No adjusted analysis was performed for this group because of the limited number of patients.

Abbreviations: CI, confidence interval, DSA; sHR, subdistribution hazard ratio.

Effect of initial immunosuppression on long term kidney transplant outcome in immunological low-risk patients

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Nephrol Dial Transplant. 2018; In press.

Abstract

Introduction

Few studies have evaluated the effect of different immunosuppressive strategies on long-term kidney transplant outcomes. Moreover, as they were usually based on historical data, it was not possible to account for the presence of pretransplant donor-specific HLA antibodies (DSA), a currently recognized risk marker for impaired graft survival. The aim of this study was to evaluate to what extent frequently used initial immunosuppressive therapies increase graft survival in immunological low-risk patients.

Methods

We performed an analysis on the PROCARE cohort, a Dutch multicenter study including all transplantations performed in the Netherlands between 1995 and 2005 with available pre-transplant serum (n=4724). All sera were assessed for the presence of DSA by a Luminex single antigen bead assay. Patients with a previous kidney transplantation, pretransplant DSA or receiving induction therapy were excluded from analysis.

Results

Three regimes were used in over 200 patients: cyclosporine/prednisolone (n=542), cyclosporine/MMF/prednisolone (n=857) and tacrolimus/MMF/prednisolone (n=811). Covariate adjusted analysis revealed no significant differences in 10-year death-censored graft survival between patients on tacrolimus/MMF/prednisolone therapy (79%) compared with patients on cyclosporine/MMF/prednisolone (82%, p=0.88) or cyclosporine/prednisolone (79%, p=0.21). However, the 1-year rejection-free survival censored for death and failure unrelated to rejection was significantly higher for tacrolimus/MMF/prednisolone (81%) compared with cyclosporine/MMF/prednisolone (67%, p<0.0001) and cyclosporine/prednisolone (64%, p<0.0001).

Conclusion

These results suggest that in immunological low-risk patients excellent long-term kidney graft survival can be achieved irrespective of the type of initial immunosuppressive therapy (cyclosporine or tacrolimus; with or without MMF), despite differences in 1-year rejection-free survival.

Introduction

Different immunosuppressive regimes are used to prevent rejection and to maintain long-term graft function in kidney transplant recipients [1]. Standard therapy in most transplant centers nowadays consists of a combination of induction therapy with an interleukin-2 receptor antagonist (IL2RA) and tacrolimus, mycophenolate mofetil (MMF) plus prednisolone [2,3]. A hallmark study that played an important role in the widespread implementation of this strategy was the ELITE-Symphony trial [4]. In this large randomized clinical trial, standard-dose cyclosporine was compared with low-dose cyclosporine, low-dose tacrolimus and low-dose sirolimus. Additional treatment for all patients consisted of MMF, prednisolone and patients treated with a low-dose prescription also received daclizumab, an IL2RA, during the first two months. Intention-to-treat analyses indicated that a low-dose tacrolimus regime was superior to all other regimes regarding renal function, acute rejection and graft-survival at 1-year follow-up [4]. After 3-year follow-up, at the end of the study, no significant difference in graft survival for both cyclosporine regimes compared with tacrolimus was observed anymore, whereas renal function and freedom from biopsy proven rejection remained superior in the tacrolimus arm [5]. Only a limited number of studies evaluated the effect of different immunosuppressive regimes on long-term outcomes [6-9]. None of these studies took the presence of pretransplant donor-specific anti-HLA antibodies (DSA), a well-known risk factor for graft loss [10,11], into account as they were primarily based on historical data. Patients at low risk of rejection, i.e. those with a first transplantation and no DSA, may require a less intensive immunosuppressive treatment compared with patients with DSA or other immunological risk markers such as retransplantation [12]. The aim of this study was therefore to assess to what extent frequently used initial immunosuppressive therapies increase graft survival in first kidney transplants without DSA in the absence of induction therapy.

Methods

Study population

We performed an analysis on the prospective PROCARE cohort, a Dutch multicenter study evaluating all transplantations performed in the Netherlands between January 1995 and December 2005 with available pretransplant serum. Detailed methods on the cohort were previously published [11]. Of note, the T-cell dependent complement-mediated cytotoxicity test was negative in all transplantations. Pretransplant sera were only recently (*post hoc*) tested for the presence of luminex defined anti-HLA antibodies. DSA were assigned for HLA-A/-B/-DQ/-DR by comparing bead specificities of the positive beads with the HLA

type of the donor on the split antigen level. Clinical data were obtained from the Netherlands Organ Transplant Registry (NOTR). Data on cold ischemia time were missing in 226 patients, historic peak panel reactive antibody (PRA) in 42 and number of HLA-A/-B/-DR mismatches in 39. We used Markov chain Monte Carlo single imputation to impute these missing values.

To construct a homogenous low-risk population, we included only recipients of a first transplantation without pretransplant DSA and who received no induction therapy. Within this low-risk population we focussed on initial immunosuppressive regimes that were used in more than 200 patients. Immunosuppressive treatment was per center's discretion, but in general target trough levels during the first months of 150-300ng/ml for cyclosporine and 10-20ng/ml for tacrolimus were pursued. If administered, then patients on cyclosporine received twice daily 1000mg MMF and patients on TAC twice daily 750mg. Prednisolone was tapered off to zero after three months or continued in a low dose (approximately 0.1mg/kg). The primary outcome was 10-year death-censored graft survival and the secondary outcome was 1-year rejection-free survival censored for death and graft failure unrelated to rejection. Rejection was defined as treatment for rejection, which in the majority of cases were biopsy-proven according to standard practice in the participating centers. Patients who were lost to follow-up over time (4.6%) were censored from survival analyses based on the recorded last date seen.

Statistical analysis

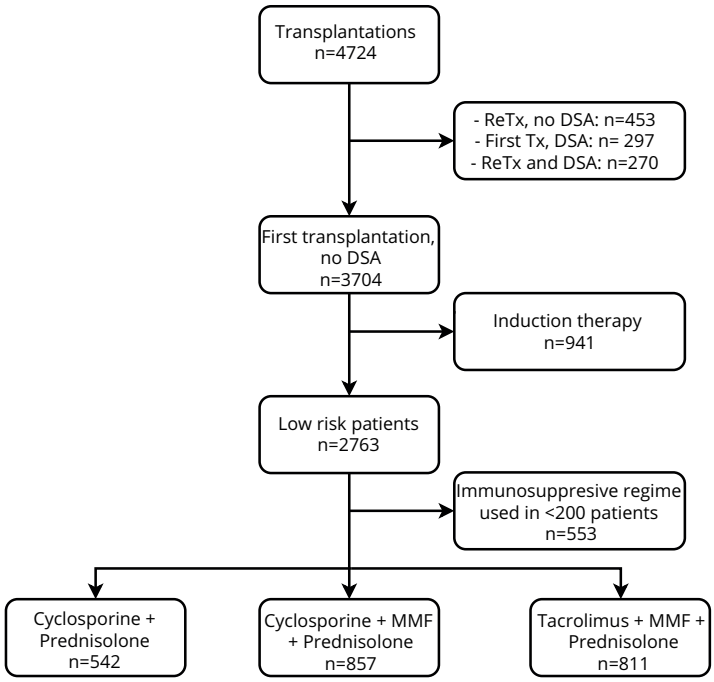
We performed an intention-to-treat analysis, evaluating the initially prescribed immunosuppressive regime without taking medication changes and adherence into account. Baseline characteristics were compared with the χ^2 test and unpaired t-test or Mann-Whitney U test as appropriate. Death-censored graft survival was compared among regimes by constructing a Kaplan-Meier curve and tested for significance with the log-rank test. In addition to restricting to immunological low-risk patients, we also adjusted for several covariates with a Cox proportional hazard model in order to limit confounding by indication [13]. The following covariates were included: recipient and donor age (both normal and quadratic), transplant center, number of HLA-A/-B/-DR mismatches on broad antigen level, historic peak PRA level, donor type and cold ischemia time for donation after brain death (DBD) and donation after circulatory death (DCD) donors. Except for transplant center, these covariates were selected because they were previously identified as risk markers for graft loss in the PROCARE cohort [11]. As the immunosuppressive treatment strategies within this observational cohort was per center's discretion, we also included transplant center. For rejection-free survival, we additionally included delayed graft function (DGF) as a covariate since patients with DGF usually undergo a surveillance biopsy 7 days after transplantation. These biopsies frequently show borderline or type IA rejection

that may not have been detected otherwise [14,15]. Statistical analyses were performed with R 3.5.1 and SAS 9.4 (SAS Institute Inc., Caru, NC).

Results

Of the 4724 transplantations with available pretransplant DSA status, 1961 were excluded because they were retransplantations, patients had pretransplant DSA and/or received induction therapy (figure 1). Within the immunological low-risks patients, three initial regimes were identified that were used in more than 200 patients: cyclosporine/prednisolone (CsA/Pred, n=542), cyclosporine/MMF/prednisolone (CsA/MMF/Pred, n=857) and tacrolimus/MMF/prednisolone (TAC/MMF/Pred, n=811). Baseline characteristics are provided in table 1. Patients on TAC/MMF/Pred were more recently transplanted and were older. Moreover their donors were older and the mean number of HLA-A/-B/-DR mismatches was higher. On the other hand, cold ischemia time for deceased donor transplantations was markedly lower and there were more living donors in the TAC/MMF/Pred group.

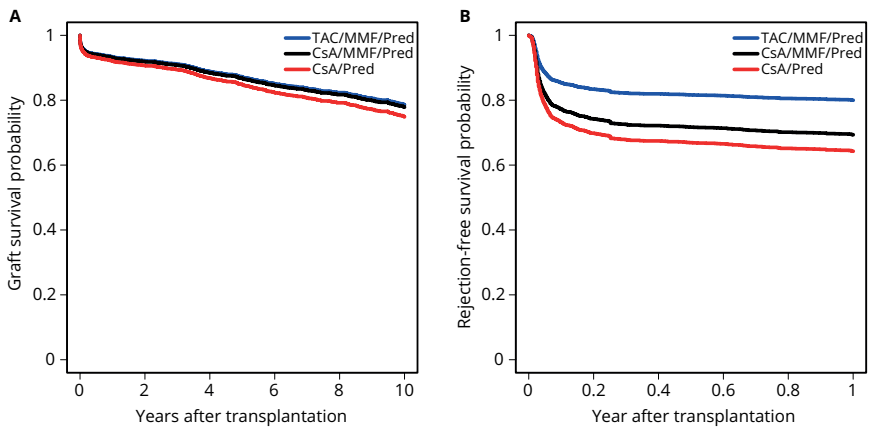
Figure 1. Flowchart for the in- and exclusion of patients.



Abbreviations: DSA, donor-specific HLA antibodies; MMF, mycophenolate mofetil; ReTx, retransplantation.

The crude 10-year death-censored graft survival was 79% in patients receiving the reference therapy (TAC/MMF/Pred) as well as in patients on CsA/Pred (p=0.69) and 82% in patients receiving CsA/MMF/Pred (p=0.08; table 2). After adjustment for several covariates in Cox multiple regression, still no significant difference in graft survival between the patients on either one of the cyclosporine based therapies and the TAC/MMF/Pred group was observed (figure 2A; table 2). The distribution of graft failure causes did not significantly differ between patients being treated with TAC/MMF/Pred and CsA/MMF/Pred or CsA/Pred (supplementary figure 1). However, one-year rejection-free survival was markedly lower in patients receiving either CsA/MMF/Pred (67%, adjusted hazard ratio (HR) for acute rejection 1.65, 95%CI 1.29-2.09) or CsA/Pred (64%, adjusted HR 1.89, 95% CI 1.47-2.42) compared with patients on the tacrolimus based regime (81%). The majority of the rejection episodes occurred within the first three months after transplantation and the difference between the regimes also emerged within this time frame (figure 2B). The percentage of patients requiring rejection treatment between month 3 and year 1, regardless of whether they received rejection treatment within the first 3 months or not, did not significantly differ among the groups (CsA/MMF/Pred =5%, CsA/Pred = 6%, TAC/MMF/Pred = 7%; overall p=0.13).

Figure 2. Cox proportional hazard estimates for graft and rejection-free survival according to initial treatment.



(A) 10-year death-censored graft survival estimates for patients on TAC/MMF/Pred did not significantly differ compared with CsA/MMF/Pred (p=0.80) or CsA/Pred (p=0.22). (B) 1-year rejection-free survival censored for death and failure unrelated to rejection estimates were significantly lower for patients on CsA/MMF/Pred or CsA/Pred compared with TAC/MMF/Pred (p<0.0001). Abbreviations: CsA, cyclosporine; MMF, mycophenolate mofetil; Pred, prednisolone; TAC, tacrolimus.

Table 1. Baseline characteristics according to initial immunosuppressive regime.

	CsA/Pred	CsA/MMF/ Pred	TAC/MMF/ Pred	*P**	*P***
Number (%)	542 (25%)	857 (39%)	811 (37%)		
Donor characteristics					
Age (years, mean ± SD)	41.7 ± 14.8	42.7 ± 15.1	46.9 ± 14.3	<0.0001	<0.0001
Gender, female – no. (%)	268 (49%)	412 (48%)	431 (53%)	0.18	0.04
Donor type – no. (%)				<0.0001	<0.0001
• Living	129 (24%)	205 (24%)	358 (44%)		
• Donation after brain death	384 (71%)	559 (65%)	260 (32%)		
• Donation after circulatory death	29 (5%)	93 (11%)	193 (24%)		
Recipient characteristics					
Age (years, mean ± SD)	43.8 ± 14.4	44.6 ± 15.0	48.0 ± 13.9	<0.0001	<0.0001
Gender, female – no. (%)	173 (32%)	341 (40%)	335 (41%)	<0.001	0.53
Peak PRA (%, median, IQR)	2 (0-9)	0 (0-5)	0 (0-4)	<0.0001	0.05
Transplant characteristics					
Cold ischemia time (hours, mean ± SD)[&]	25.0 ± 7.1	21.9 ± 6.9	19.5 ± 7.1	<0.0001	<0.0001
HLA-A, -B, -DR broad mm (mean ± SD)	2.0 ± 1.3	2.1 ± 1.5	2.6 ± 1.5	<0.0001	<0.0001
Transplant year (mean ± SD)	1997 ± 2	1999 ± 2	2002 ± 2	<0.0001	<0.0001

Abbreviations: IL2rMoAb, anti-Interleukin-2 receptor monoclonal antibody; mm, mismatches; PRA, panel reactive antibodies.

[&]For deceased donors only.

* Cyclosporine + Prednisolone (CsA/Pred) compared with Tacrolimus + MMF + Prednisolone (TAC/MMF/Pred).

** Cyclosporine + MMF + Prednisolone (CsA/MMF/Pred) compared with Tacrolimus + MMF + Prednisolone.

Table 2. Transplant outcomes compared among different initial immunosuppressive regimes.

Regime	Crude 10-year Graft survival	Graft failure - unadjusted HR (95% CI)	Graft failure - adjusted HR* (95% CI)
TAC/MMF/Pred	79%	Reference therapy	
CsA/MMF/Pred	82%	0.81 (0.65-1.02)	1.02 (0.77-1.36)
CsA/Pred	79%	1.06 (0.83-1.35)	1.21 (0.90-1.61)
	Crude 1-year rejection-free survival	**Rejection within year 1 - unadjusted HR (95% CI)**	**Rejection within year 1 - adjusted HR[#] (95% CI)**
TAC/MMF/Pred	81%	Reference therapy	
CsA/MMF/Pred	67%	1.89 (1.55-2.30)	1.65 (1.30-2.10)
CsA/Pred	64%	2.10 (1.69-2.60)	1.96 (1.53-2.51)

Abbreviations: CI, confidence interval; CsA, cyclosporine; HR, hazard ratio; MMF, mycophenolate mofetil; Pred, prednisolone; TAC, tacrolimus.

*Adjusted for: center, recipient age (^{2}), donor age (^{2}), donor type, cold ischemia time for DBD and DCD donors, peak PRA and number of HLA-A/B/DR mismatches.

[#]Adjusted for: center, recipient age (^{2}), donor age (^{2}), donor type, cold ischemia time for DBD and DCD donors, peak PRA, number of HLA-A/B/DR mismatches and delayed graft function.

Discussion

The results of this study show that irrespective of the type of initial immunosuppressive therapies (cyclosporine or tacrolimus; with or without MMF), excellent long-term graft survival can be achieved in immunological low-risk patients compared with the general graft survival of transplantations performed within the same period [16]. Patients who were treated with cyclosporine showed higher incidences of acute rejection, which is consistent with observations from the ELITE-Symphony trial [4] and others [17,18]. If adequately treated, then early acute rejection is usually reversible and has only limited effect on long-term graft survival [19,20]. We think that the lack of differences in graft survival despite the higher incidence of acute rejection in the cyclosporine treated patients can be explained by the fact that the majority of the rejection episodes occurred within the first three months. Previous clinical trials comparing tacrolimus and cyclosporine in different combinations mostly had a short follow-up and showed discrepant results, though they generally did not indicate a difference in long-term graft survival [5,17,21-23]. Several large observational studies also showed no differential effect of initial immunosuppressive therapies on mid- and long-term graft survival [6-9,24].

This analysis was conducted on a large multicenter prospective cohort with at least 10-year follow-up and relatively few missing data. Unique for this analysis is the availability of information on pretransplant DSA status. In this cohort, the choice of immunosuppressive therapy was not influenced by pretransplant DSA status because luminex single antigen bead testing for anti-HLA antibodies was not being performed at the time of transplantation. We resigned from performing separate analysis on immunological high-risk patients (DSA positive and/or retransplantations) since numbers were too low for precise estimates, and reliable adjustments for potential confounders and thus prevent spurious findings. Induction therapy was not routinely given in the Netherlands at the time of transplantation of this cohort and could thus introduce confounding by indication. We, therefore, decided to exclude patients receiving induction therapy leaving a study population as homogenous as possible.

An important limitation of this study is that clinical practice evolved during the study period as reflected by the close relation between immunosuppressive treatments and era-dependent changes in donor and recipients. Patients receiving TAC/MMF/Pred were more recently transplanted and consequently donors were older and the mean number of HLA mismatches was higher, both risk markers for graft loss [16,25]. On the other hand, cold ischemia time was shorter and there were more living donors in this group [26]. We adjusted for all these factors and also for historic peak PRA and transplant center in Cox multiple regression to reduce the potential bias induced by these differences.

Transplant year, continuous or stratified, did not influence the relation between initial immunosuppression and graft survival and was therefore not included as a covariate in the Cox multiple regression model. Other limitations of this study are that we could not account for drug dosages and trough levels (if applicable) and had limited follow-up data [26]. For acute rejection, we only had information on whether a patient received treatment for acute rejection. Unfortunately, we do not have information on the type and severity of rejection. In addition, no information on *de novo* DSA development was available. Several lines of data indicate that tacrolimus is associated with a lower risk of *de novo* DSA compared with cyclosporine, whereas the impact of MMF remains controversial [27,28]. We cannot rule out that patients with CsA/Pred developed more often *de novo* DSA than patients on TAC/MMF/Pred. Lastly, we do not have reliable information on incidence rates of adverse events such as infections and malignancies. Regarding opportunistic infections, other studies suggested that the incidence of BK virus infections is the highest in patients on triple therapy, in particular in patients receiving both tacrolimus and MMF [21,29,30]. Moreover, in some studies treatment with MMF was also associated with increased risk of CMV infections [21,31,32]. As screening for and management of BK nephropathy has improved since the early 2000s [33], we cannot rule out that suboptimal management has contributed to the lack of difference in graft survival between patients on TAC/MMF/Pred compared with CsA/Pred.

Taking these limitations into consideration, we suggest that in selected patients at immunological low-risk but at high risk of side effects, minimization of initial immunosuppressive therapy might be a valid alternative. Elderly patients without DSA may particularly benefit from a minimized, age-adapted immunosuppressive therapy. This group is prone to the development of adverse events [34,35] and is less susceptible to acute rejection because of immunosenescence [36]. We did not analyse other forms of reduced immunosuppression than CsA/Pred because of limited patients numbers. Nonetheless, our data could suggest that a combination of TAC/Pred might also be adequate in these patients. Limited information on this combination as initial therapy is available. A randomized clinical trial including the first and the second deceased donor transplantations, indicated a higher incidence of acute rejection in patients on TAC/Pred compared with TAC/MMF/Pred, but no difference in 1-year graft survival [32]. Alternative minimization strategies to reduce the side effect burden in immunological low-risk patients are dosage reduction or initial triple therapy followed by withdrawal of one or two immunosuppressant drugs within the first months posttransplantation [20,37]. In line with these considerations, a few initiatives are being undertaken to set up prospective clinical trials investigating minimisation strategies in elderly transplant recipients (ClinicalTrials.gov: NCT02453867). These clinical trials should not only focus on acute rejection and graft survival but also include infections and de novo DSA development in their outcome measures.

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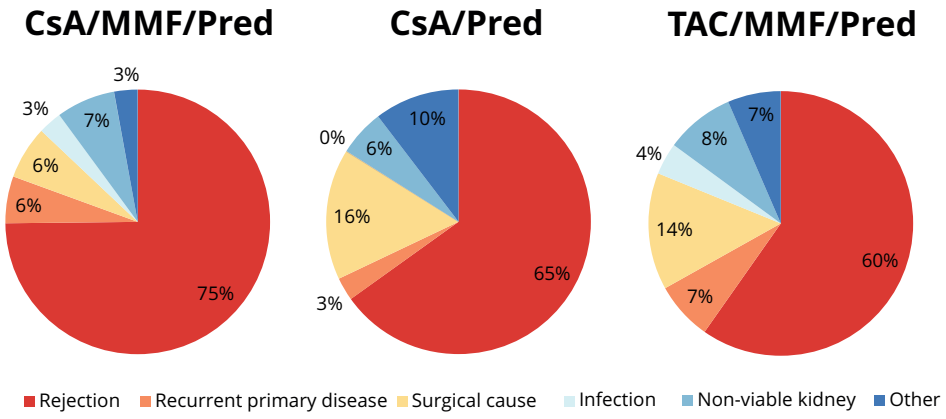
Supplementary material

Supplementary figure 1. Failure causes per initial immunosuppressive regime.



Failure causes as reported to Eurotransplant did not differ significantly between patients on TAC/MMF/Pred and CsA/MMF/Pred or CsA/Pred (overall p=0.10 and p=0.13 respectively). Surgical cause includes graft thrombosis.

Clinical value of non-HLA antibodies in kidney transplantation: still an enigma?

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Abstract

HLA antibodies play a major role in the recipient's immune response against the renal allograft and are an established risk marker for antibody-mediated rejection and subsequent impaired graft survival. Evidence originating from HLA-identical donor-recipient pairs indicates that non-HLA antibodies may play a role as well. Numerous non-HLA antibodies have been identified in renal organ transplantation, directed against a heterogeneous subset of both allo- and autoantigens including MHC Class-I-related chain A (MICA) and Angiotensin II type 1 receptor (AT_1R). In this review, we will discuss the mechanisms predisposing to non-HLA antibody formation, the possible synergy with HLA-antibodies in their pathologic potential and the mechanisms involved in allograft damage. Furthermore, an overview of the identified non-HLA antibodies and antigens and their relation with rejection and graft survival will be provided.

Introduction

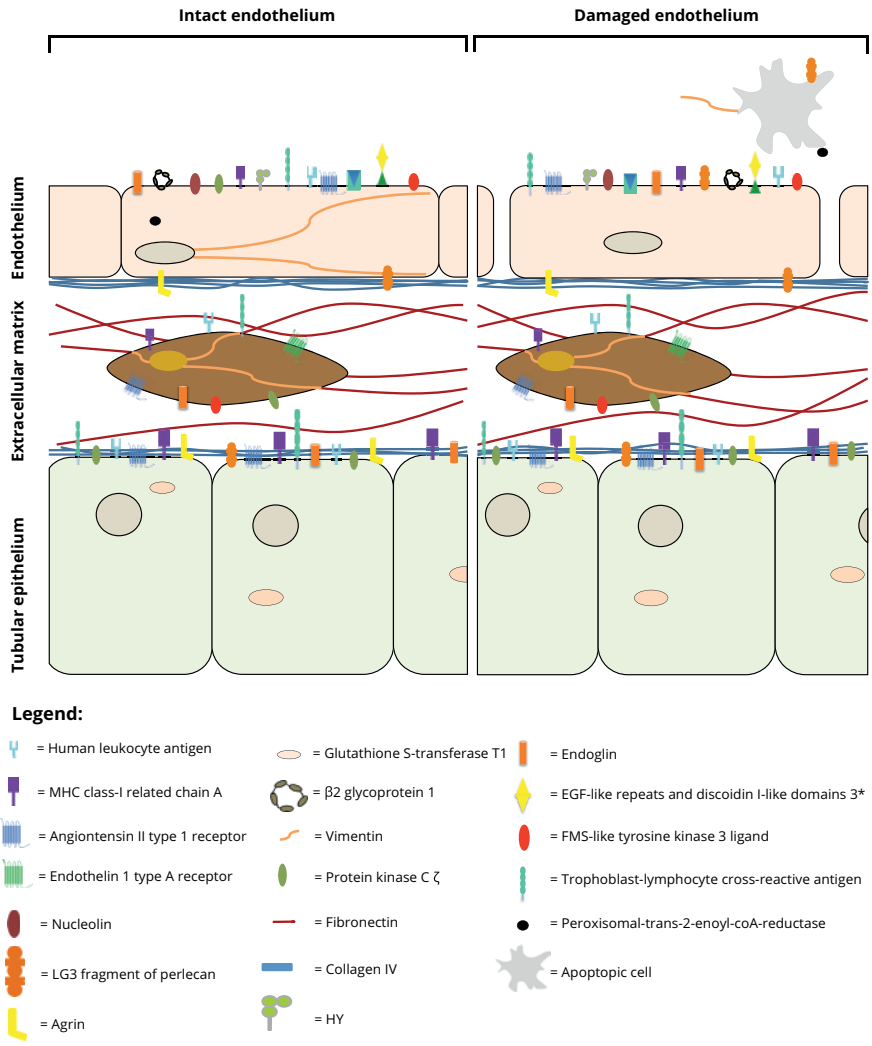
Advances in organ preservation, surgical techniques and immunosuppressive agents have led to markedly improved short-term outcomes, and long-term kidney graft survival is also starting to improve. However, acute and chronic rejection continue to be important causes of graft failure [1-4]. Major histocompatibility complex (MHC) molecules expressed on donor cells are the main targets of the recipient's immune response and are involved in cellular and humoral rejection. The presence of donor-specific HLA-antibodies is a major risk marker for antibody-mediated rejection (AMR) and has a detrimental effect on graft survival [5-10]. Non-HLA antibodies also play an important role in transplant rejection and increasing numbers of antibodies have been identified over the past years. This was highlighted by Opelz et al., who found that among 4000 recipients of HLA-identical sibling transplants, the panel reactive antibody (PRA) reactivity was strongly associated with long-term graft loss [11]. These results suggest that PRA reactivity is associated with increased alloresponse to the graft including to non-HLA antigens. Several reviews relating to non-HLA antibodies have been written including overviews on the role of specific non-HLA antibodies and mechanistic concepts of humoral autoimmunity in solid organ transplantation [12-16]. The number of published articles on this topic is still increasing, several papers have been published recently [17-19]. In this review we will present the current concepts of the mechanisms involved in non-HLA antibody formation, their proposed working mechanisms and provide an overview of the identified non-HLA antibodies in renal transplantation and their role in rejection and subsequent allograft loss.

Mechanisms involved in antibody formation

Non-HLA antibodies can be directed against auto- or alloantigens and be present either pretransplantation or *de novo* formed posttransplantation. Figure 1 provides an overview of the localization of identified antigens within the peritubular capillary, one of the main sites for AMR, both in quiescent state and following endothelial damage. Antibody formation against polymorphic alloantigens such as MHC Class-I-related chain A (MICA) was originally thought to be related to the same sensitizing events as in HLA-antibodies (previous transplantations, blood transfusions and pregnancies) [20,21]. However, several studies do not report an association with sensitizing events, therefore other mechanisms involved in stimulation of antibody formation should be considered [22-24]. For MICA, it was shown that an A5.1 mutation in the donor, that is related to the MICA*008 allele, is associated with a strongly increased MICA expression on donor endothelial cells compared with wild type donors and therefore these mutated MICA

molecules are important targets for antibody formation [25]. Furthermore, mismatching on certain amino acid residues leads to increased MICA-antibody formation and it can be that based on the 3d-structure of MICA, these structures are more accessible for antibodies [21].

Figure 1. Localization of non-HLA antigen targets within the peritubular capillary in quiescent state en following endothelial damage.



For autoantibody formation, several triggering events are proposed that can attribute to stepwise loss of self-tolerance. Firstly, inflammation can lead to upregulated antigen expression and altered antigen processing and proteolysis with subsequent rearrangement of cytosolic proteins and exposure to cryptic epitopes [12,26-28]. Furthermore, posttranslational modification, oxidative stress and apoptosis may result in the formation of neoantigens [12,29]. Finally, close relatedness of infectious agents and self-peptides could lead to cross-activation of autoreactive B and T cells [12,30,31]. Follicular helper T cells (Tfh) play a crucial role in B cell selection directed against alloantigens and prevent the formation of autoreactive B cell clones. Deregulation of Tfh cells is likely to be involved in humoral autoimmunity through impaired B cell selection [32].The loss of self-tolerance may be further facilitated by impaired deletion of immature autoreactive B cells through increased Th17 generation in response to the allograft and/or donor HLA [33,34].

Mechanisms of non-HLA antibody-mediated graft damage

It is hypothesized that HLA- and non-HLA antibodies have a synergistic effect. While HLA-antibodies can evoke endothelial damage and subsequent exposure to autoantigens resulting in autoantibody formation, an inflammatory response induced by non-HLA antibodies could in turn upregulate HLA expression and thereby make the allograft more prone to an alloimmune response [15]. This hypothesis is supported by several studies that showed that patients with both HLA and non-HLA antibodies had lower graft survival rates compared with patients with either one of them [35-37]. In line with this, another study suggested that patients who developed acute rejection had more often both donor-specific HLA and anti-angiotensin II type 1 receptor (AT_1R) antibodies at time of transplantation than only one of them [38]. A comparable phenomenon has been observed in heart transplantation in relation to the occurrence of rejection [39]. Furthermore, serial antibody monitoring in transplant patients has revealed patterns of antibody development, including HLA antibodies preceding non-HLA antibodies and a tandem development [36]. Nonetheless, others question this putative linkage as they found no association between non-HLA antibodies and graft survival in patients with simultaneous HLA-antibodies [22].

Most of the identified autoantigens are not exclusively expressed in the kidney, but ubiquitous throughout the body including in most other solid organs [40]. This suggests that these antibodies act through highly specific mechanisms and may require a local co-stimulating event, like HLA antibody binding or ischemia reperfusion injury, in order to induce response. It is unlikely that non-HLA antibodies can directly induce major graft damage

since hyperacute rejection induced by these antibodies rarely occurs [41]. Several mechanisms are hypothesized to play a role in non-HLA antibody-mediated graft injury. It is of interest to note that this is mainly based on knowledge on anti-HLA antibodies and on the effector mechanisms of the specific antigen targets, as animal and *in vitro* studies on the pathogenic role non-HLA antibodies in solid organ transplantation are scarce. The presumed antigen-specific mechanisms for some antibodies will be discussed in the next paragraph; here we will review general mechanisms of antibody-mediated endothelial damage. Firstly, IgM and some IgG antibodies can fix C1q and thereby activate the complement cascade, which leads to the formation of membrane-attack complexes. IgG1 and IgG3 subclasses are the most efficient in fixing C1q, while IgG2 and IgG4 antibodies exhibit almost no complement fixation [42]. Lytic amounts of complement-fixing antibodies and formed membrane-attack complexes can cause hyperacute rejection, which is rarely observed nowadays due to crossmatching. On the other hand, sublytic exposure results in endothelial activation which induces amongst others changes in cytoskeletal formation, release of heparan sulfates and a procoagulant and proinflammatory state [43,44]. Antibody binding can also induce endothelial cell activation and a subsequent immune response independent of complement, for instance through activation of intracellular signaling cascades [45-47]. Besides, antibodies can induce lysis of target-cells with membrane bound antibodies through activation of natural-killer cells, a process called antibody-dependent cell-mediated cytotoxicity [48].

Non-HLA antibodies and their clinical relevance in renal transplantation

The non-HLA spectrum covers a broad range of allo- and autoantigens. This is illustrated by a study by Li et al. who tested pre- and posttransplant samples of pediatric renal transplant patients for reactivity against over 5000 protein targets selected on appearance in the kidney in a ProtoArray and reported a response to 61% of the targets on average. The highest reactivity was seen against antigens expressed in the cortex and renal pelvis such as Stathmin-like 3, a protein expressed on pelvic epithelial cells [49]. This underlines that non-HLA antibodies are not exclusively directed against targets on the endothelium. Additionally, Porcheray et al. have suggested that antibodies produced by B-cell clones are rather polyreactive instead of directed against a single antigen-target. They isolated B-cell clones from a recipient suffering from AMR and discovered that some of these B-cell clones produced antibodies not only reactive to HLA and MICA, but also to autoantigens including DNA and cell cytoplasmic structures [50]. In the following paragraphs non-HLA antibodies that were studied in at least two cohort studies will be discussed.

Anti-endothelial cell antibodies

The endothelium in the allograft is the initial contact site between donor and recipient and therefore functions as an important defense barrier [43,51]. Anti-endothelial cell antibodies (AECAs) are directed against a variety of antigenic targets expressed on endothelial cells including AT_1R, vimentin and peroxisomal trans-2-enoyl-CoA reductase (PECR) [52-54]. Recently, four novel targets were identified through protein analysis of AECAs eluted from endothelial precursor cell (EPC) crossmatch tests: endoglin, EGF-like repeats and discoidin I-like domain 3, intercellular adhesion molecule 4 and Fms-like tyrosine kinase 3 [17]. Immunoprecipitation of AECAs directed against HUVECs followed by mass spectrometry has also identified several potential new targets including human keratin 1 and eukaryotic translation initiation factor 2A [55]. Based on the observation that AECAs reactive to EPCs are enriched for IgG2 and IgG4, it is suggested AECAs act rather through complement-independent endothelial cell activation and disturbing vascular integrity [56,57]. This is in line with a study on AECAs directed against donor-matched rat-heart endothelial cells, showing that only MHC-mismatched allosera could induce C3 deposition on endothelial cells and a positive complement-dependent crossmatch, whilst MHC-matched allosera with AECAs could not [58].

Anti-endothelial cell antibodies can be detected amongst others with flow cytometry, ELISA, indirect immunofluorescence and high-density protein array [17,59-61]. Most assays use either human umbilical vein endothelial cells (HUVECs) or EPCs isolated from peripheral blood as an antigen substrate. Nowadays, commercial assays for endothelial cell crossmatching are available that use magnetic coated beads against angiopoietin receptor Tie-2 to isolate EPCs (ECXM and XM-ONE®) [62,63]. However, the current detection methods have several limitations. Various endothelial cells have different antigen expression patterns and may respond different to activation. The importance of using endothelial cells derived from vessels of appropriate size has been underlined by several studies including work on AECAs in various small and large vessel diseases [64,65]. Moreover, it was shown that upon binding of HLA class I antibodies on aortic, human umbilical and dermal microvasculature endothelial cells, the induction of P-selectin, involved in recruitment of leukocytes, varied amongst cell types [66]. Some studies use immortalized endothelial cell lines instead of primary cells for their assays. A comparison of HUVECs and EA.hy926, a commonly used immortalized HUVEC cell line, showed that EA.hy926 cells have a high similarity with primary endothelial cells but show differences on expression levels of certain endothelial markers and express a large number of additional genes mainly related to cell cycle and apoptosis [67]. Circulating EPCs isolated from peripheral blood enable to test for donor-specific AECAs. However it's not clear whether these latter cells reflect the properties of those endothelial cells present in the graft [68]. It has been shown that EPCs isolated through

anti-Tie-2 magnetic beads lack some characteristic endothelial surface markers including CD31 and CD34 [62,68]. Therefore, antibodies reactive against antigen targets on EPCs or HUVECs may not react against antigens expressed on renal microvascular endothelial cells. Finally, donor-specific HLA reactivity should be ruled out as both EPCs and HUVECs express HLA [17,69].

Numerous studies have explored the influence of AECAs on clinical outcomes. The largest cohort study thus far, evaluated pretransplant sera of 392 renal transplant patients using a cellular ELISA with HUVECs. AECAs were present in 15.8% of all patients and were associated with an increased risk of acute rejection, mainly vascular rejection grade IIb or AMR, within the first six months posttransplantation (OR 2.47, p=0.01) [60]. Xavier et al. included 160 posttransplant patients and used EPCs isolated from non-donor peripheral blood for flow cytometry crossmatching. Forty percent tested positive for AECAs and this was associated with an increased risk for rejection (RR 3.87; 2.15-6.96) [61]. In a study by Sun et al. 226 recipients of a first and cadaveric renal allograft were tested pre- and posttransplantation for the presence of AECAs against HUVECs through indirect immunofluorescence. Twenty-three percent was positive pretransplantation and 9.8% developed *de novo* antibodies. Although pre-existing antibodies showed no association with acute rejection nor graft survival, *de novo* antibodies were associated with a higher incidence of early acute rejection and steroid resistant rejection and lower graft survival [59]. Several other studies have also suggested that AECAs are a risk marker for acute rejection [70-73]. In line with the findings by Sun et al., Zinger et al. used donor-specific EPCs for their crossmatch and concluded that pretransplant AECAs were not associated with an overall poorer graft outcome or number of acute rejection episodes [74]. Furthermore, a replication of the study by Sun et al. in a Caucasian population showed no relation between pretransplant or *de novo* AECAs and rejection or graft survival. The absent relation between *de novo* AECAs and rejection episodes should be interpreted with caution because only four patients had *de novo* AECAs [75]. The authors give several other explanations for the conflicting results, including a relatively low number of highly sensitized patients, routinely induction therapy and protocol versus for-cause biopsies [74,75].

In summary, multiple studies suggest that AECAs are a risk marker for acute rejection mainly in the early posttransplant period [59-61,70,73]. Major limitations of AECAs are use of non-kidney derived endothelial cells in most detection techniques, discrepancies in detection by different techniques such as flow cytometry and cellular ELISA and lack of knowledge on antigen specificity [17,76,77]. Therefore, it might be more sensible to look at specific antigens that are expressed on the renal microvasculature for sure.

Angiotensin II type 1 receptor antibodies

The AT_1R is a transmembrane G-protein coupled receptor that exerts the effects of its endogenous ligand angiotensin II, including blood pressure regulation and the water- and salt balance [15]. AT_1R-stimulating autoantibodies are directed against two epitopes on the second extracellular loop of the receptor and induce phosphorylation of the ERK1/2 pathway in endothelial cells and increase the binding activity of transcription factors nuclear factor-κB and activator-protein 1. The ERK 1/2 pathway has numerous functions including the regulation of cell adhesion, migration, survival and proliferation [52,78]. Subclass determination indicated that only complement-fixing subclasses IgG1 and IgG3 exert an agonistic effect on the AT_1R [52]. An ELISA-based kit is available to test for these antibodies (EIA-AT_1R, OneLambda, Canoga Park, CA, USA). Anti-AT_1R antibodies are considered to play a role in the pathophysiology of several vascular diseases including pre-eclampsia, malignant hypertension and systemic sclerosis [79,80]. Dragun et al. were the first to describe the presence of these IgG1 and IgG3 subclass autoantibodies in almost half of a group of renal transplant patients with steroid refractory vascular rejection and malignant hypertension [52]. Following AT_1R-antibody infusion, transplanted rats showed signs of endarteritis and intravascular infiltrates in their allografts, while native kidneys in uninephrectomized rats showed no abnormalities upon biopsy [52]. These results suggest that AT_1R antibodies cannot induce rejection on their own but require an alloimmune response.

A retrospective assessment of pre- and posttransplant sera from 351 patients showed that patients with AT_1R had abnormal biopsy results more often. In multivariate analyses only AT_1R antibodies that developed *de novo* were significantly associated with a decreased graft survival for the entire cohort (HR 5.35, $p<0.0001$). A subanalysis on 134 patients with abnormal biopsies revealed that both persistent and *de novo* AT_1R antibodies were significantly associated with graft failure. In these patients, the co-occurrence of posttransplant AT_1R antibodies and donor-specific anti-HLA antibodies was associated with the poorest graft survival [36]. A second large study including 599 patients showed that pretransplant AT_1R positive patients had a significantly increased risk of graft loss from three years post-transplantation onwards and acute rejection within the first four months. Approximately half of the AT_1R positive patients with acute rejection suffered from AMR. However, it should be noted that only 4 of the 22 patients with acute rejection and pretransplant AT_1R antibodies still had positive AT_1R antibodies at time of rejection. The authors suggest that lower antibody titers during rejection could be due to antibody adsorption in the graft [38]. However, graft failure related to other factors like donor-specific HLA antibodies should be considered. Finally, Banasik et al. prospectively assessed pre- and posttransplant sera of 117 patients and reported that positive antibodies pretransplantation and at month 3, 6 and 12 were significant risk markers for graft failure. There was no significant association

with acute rejection episodes, but pretransplant antibodies were a risk marker for AMR and grade IIb rejection in subanalysis [81]. In conclusion, these studies all indicate that AT_1R-antibodies are associated with an increased incidence of graft failure. Remarkably, despite the fact that all studies used the same ELISA-based assay, the prevalence of AT_1R-antibodies pretransplantation ranged from 17-47%. This could be partly attributed to differences in used cut-off values.

Endothelin-1 type A receptor antibodies

Vascular endothelium and renal epithelial cells produce endothelin-1 which plays a role in blood pressure regulation and can bind to two G-protein coupled receptors: endothelin type A or B receptor (ET_AR or ET_BR). Binding of endothelin-1 to ET_AR on underlying vascular smooth cells induces vasoconstriction and has mitogenic and pro-inflammatory effects including macrophage infiltration and the production of inflammatory cytokines, thereby disrupting vascular integrity [82-85].

In systemic sclerosis patients, activating autoantibodies against ET_AR frequently occur coincidently with AT_1R autoantibodies [79]. Evidence on the role of anti-ET_AR antibodies in renal transplant patients is limited. In a cohort of 116 patients, anti-ET_AR antibodies were present in almost half of the pretransplant samples and were associated with higher serum creatinine values within the first year. Although there was no difference in overall rejection episodes, mild to severe intimal arteritis occurred more often in ET_AR positive patients [86]. The prevalence of anti-AT_1R and anti-ET_AR was markedly lower in a cohort of 35 patients with stable graft function at five years after transplantation: antibodies were present in 17% and 11%, respectively [87]. In patients that underwent a for cause biopsy (n=65), anti-AT_1R and/or anti-ET_AR antibodies were present in 10.7% and this was associated with a higher incidence of graft loss [88]. The large dispersion in prevalence of pre- and posttransplant anti-ET_AR antibodies could partially be attributed by different cut-off values [86]. These studies that were all performed by the same group, justify continuing research on the role of ET_AR-antibodies in renal transplantation.

MHC class I related chain A antibodies

The MICA gene locus is located on chromosome 6 in close linkage-equilibrium to the HLA-B locus and is highly polymorphic with over 100 alleles identified and still more are being identified [89,90]. MICA is a ligand for the NKG2D receptor that is expressed on natural killer cells and γδ and αβ CD8+ T cells and is involved in recognition and eradication of infected and transformed cells [22,25]. Cellular stress conditions including infection and hypoxia induce MICA expression on endothelial cells and other cells including fibroblasts [91,92]. MICA antibodies are polyspecific, positive sera react against five different MICA antigen specificities on average [18]. The pathologic potential of MICA antibodies could be

mediated through complement fixation. In a recent study, 23% of the sera from patients with preformed MICA antibodies could fix C1q. Their cytotoxic capacity was confirmed in a complement-dependent cytotoxicity assay using activated lymphocytes [18]. As MICA is not expressed on resting lymphocytes, they cannot be detected with classical lymphocyte crossmatching [14]. Two different single antigen Luminex assays are available that detect antibodies against 28 and 11 MICA antigen specificities covering approximately 94-98% and 90% of the antigens present, respectively (LIFECODES® LSA-MIC and LABScreen MICA Single Antigen) [93].

MICA antibodies have been extensively investigated in over 35 renal transplant studies thus far. We will confine our review of the literature to the largest cohorts that included at least 400 patients. Yet, a limitation is that most of them did not determine donor-specificity. In 2007, a study that evaluated 1910 pretransplant sera for the presence of MICA antibodies (11.4%) reported that antibodies were associated with a lower one-year graft survival (HR 1.63, p=0.023). Subsequent multivariate analysis revealed that this was only significant in low immunological risk transplantations: first transplants, zero or one HLA mismatches or patients without panel reactive antigen reactivity [22]. In approximately 3000 posttransplant samples from patients without HLA antibodies and with a functioning graft on month six, MICA antibodies were found in 5.4%. The results indicated that MICA antibodies were an independent risk marker for graft loss on year one and four [94]. Sanchez-Zapardiel tested for pretransplant MICA antibodies with a screenings assay for HLA and MICA antibodies and found MICA-antibodies in 7.2%. Partly in line with previous results, they reported that pretransplant HLA-MICA+ patients had the highest risk for acute rejection within the first three months (OR 5.07; p=0.049), beyond this period there were no significant differences in rejection episodes and graft survival. However, it should be taken into account that there were only 25 HLA-MICA+ patients in a cohort of 727 patients total and collinearity between PRA and HLA/MICA status may have resulted in an overestimation of the effect [23]. Further analysis of this cohort, revealed that only half of the initially MICA positive patients remained positive after using a single antigen assay. MICA antibodies turned out to be donor-specific in 53% of cases and donor-specificity did not correlate with allograft function on month three [18]. In contrast to the previous studies, Lemy et al. found pre- and posttransplant antibodies in 13.9% and 5.4% of patients and did not find a correlation with acute rejection episodes or graft survival. Posttransplant MICA antibodies were associated with a higher incidence of chronic rejection in univariate analysis. Unfortunately no multivariate analysis could be performed because of the low number of events [20,93]. Donor-specificity was also taken into account by Cox et al. who reported that 76.5% of MICA antibodies were donor-specific (5.7% of total cohort) and this was only correlated with kidney function on year two and not on one and three years [21].

The studies differ on numerous aspects that could have contributed to the conflicting results. Firstly, not all studies investigated the same number of antigen specificities as they used different Luminex assays and the inter-test concordance is an issue. In a cohort of 779 patients, the LIFECODES® LSA-MIC and LABScreen MICA Single Antigen disagreed in 6.2% of all patients on whether a patient was MICA-antibody positive or negative [20]. Lemy and Cox required at least two different positive assays in order to define a sample as positive or only one in case a certain MICA specificity could only be detected by the LIFECODES® LSA-MIC assay [20,21,93]. Furthermore, since most studies did not take donor-specificity into account it is possible that differences in the incidence of donor-specific antibodies may have contributed to different results. Another possible explanation is the differences in induction therapy and immunosuppressive regimens between studies. Lemy et al. argue that their patients were more heavily immunosuppressed and this may have diminished the harmful effect of MICA antibodies [20]. Finally, the studies also differ on follow-up time. It could be that C1q fixing antibodies contribute to an early detrimental effect, while the long-term outcome may be more strongly affected by other factors including HLA antibodies.

Despite the heterogenic study designs and partly conflicting results, the adverse effect of MICA antibodies, regardless of donor-specificity, on graft survival in two independent multicenter studies including 1900 and 3000 patients each cannot be overlooked [22,94]. A negative impact of MICA antibodies on transplant outcomes is further supported by a systematic review by Lu et al. that was published in 2011 and included 13 kidney transplant studies. In studies with a follow-up time of at least two years, pooled analysis revealed a decreased graft survival and higher acute rejection rate but not chronic rejection in patients with posttransplant MICA-antibodies. No meta-analysis on pretransplant antibodies could be performed due to the low number of studies assessing pretransplant MICA antibodies at that time [95].

Other non-HLA antibodies

Antibodies reactive against several other non-HLA antigens have been associated with impaired outcomes following kidney transplantation. However, most of these studies are hampered by small sample sizes and case-control designs and therefore warrant further validation. An overview of the clinical studies on other non-HLA antibodies is provided in Supplementary Table 1.

Anti-vimentin antibodies have been studied in two case-control studies. Mean anti-vimentin IgG levels were found to be elevated in patients with interstitial fibrosis and tubular atrophy compared with stable individuals and patients with previously failed renal allografts also had higher antibody levels compared with patients with primary end-stage renal disease

[27,53]. Another non-HLA antibody that has been studied in renal transplant patients is directed against LG3, a C-terminal fragment of perlecan, that is involved in obliterative vascular remodeling during rejection [96]. It was shown that patients with acute vascular rejection had higher pre- and posttransplant anti-LG3 levels compared with patients with stable allograft function or acute tubulo-interstitial rejection [37]. Promising results have also been published on anti-apoptopic cell antibodies in a cohort of 300 kidney transplant patients. The authors found that even after adjusting for potential confounders including HLA class I and II and MICA reactivity, pretransplant IgG antibodies reactive to apoptotic cells were associated with an increased risk of allograft loss beyond one year posttransplantation (HR 2.271, $p<0.001$). The vast majority of serum IgG antibodies reactive to apoptotic cells turned out to be of complement-fixing IgG1 and IgG3 subclasses [97].

Conclusion

The importance of non-HLA antibodies in renal transplantation in addition to classical HLA-antibodies is increasingly being acknowledged. Multiple non-HLA antibodies have been identified in kidney transplant patients and proteomic approaches are enabling us to identify even more potential antigen targets. Most of these antigen targets are ubiquitously expressed throughout the body and some of these antibodies including AECAs and AT_1R-antibodies are associated with a wide range of vasculopathies [98]. This suggests that autoantibodies act through highly specific pathological mechanisms and may require changes in the microenvironment in order to induce a local response in the renal allograft [99]. One such inflammatory trigger could be HLA-antibody binding on the donor endothelium. Another inflammatory trigger that may attribute to non-HLA antibody-mediated graft damage is ischemia-reperfusion injury [100]. This is nicely illustrated by an animal model on passive anti-LG3 transfer in mice with a fully MHC matched aortic allograft. Vascular injury and endothelial apoptosis promote the release of LG3. Passive anti-LG3 transfer in mice with a nonischemic allograft resulted only in moderated neointima formation, while mice with an ischemic allograft showed C4 deposition, increased NK cell infiltration and obliterative vascular remodeling [37]. Irrespective of the site specificity of non-HLA antibodies, the mechanisms by which these antibodies may induce rejection are not fully understood as well. Though, there are a few animal studies on different types of solid organ transplantation indicating that non-HLA antibodies are capable of inducing rejection on their own or can aggravate anti-HLA antibody-mediated rejection [52,99,101,102]. The most extensively studied non-HLA antibodies in renal transplantation are AECAs, AT_1R and MICA antibodies. Although several studies showed an increased incidence of acute rejection in patients with AECAs, no distinct association with graft failure has been reported [59-61,70-73]. The focus seems to have shifted towards the identification of novel AECA

targets and clinical studies on antibodies directed against specific endothelial antigens such as AT_1R [17,55]. Several clinical studies on anti-AT_1R antibodies found an association with decreased graft survival and passive AT_1R-antibody transfer in transplanted mice resulted in vascular rejection, thereby supporting a causative role [36,38,52,81]. The results of studies on MICA antibodies are somewhat more conflicting. Two large multicenter studies showed that MICA antibodies were associated with a higher incidence of allograft failure in patients without HLA antibodies or that were otherwise at low immunological risk [22,94]. Yet, other smaller, more recent studies failed to show an association with unfavorable graft survival [20,23,93]. This could reflect that MICA antibodies are a particular risk marker for certain patient populations, whilst graft survival might be more strongly influenced by alternative factors in other populations.

The determination of the exact clinical relevance of non-HLA antibodies in renal transplantation is impaired by highly heterogenic study designs including differences in testing methods, immunosuppressive regimens and outcome measures. Considering the technical difficulties of current non-HLA antibodies assays and the large variation in reported incidences of antibodies even with the same assays, continuous efforts to develop reliable and sensitive diagnostic tests are essential. Besides, measuring a panel of antibodies instead of one antibody at a time will provide valuable information regarding the role of non-HLA antibodies in rejection and could eventually help identifying different risk profiles for rejection and impaired graft survival. A new Luminex-based assay that will test for multiple non-HLA antibodies at once, is currently under development and will be validated in a large multicenter cohort [103].

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Supplementary material

Supplementary Table 1. Summary of studies on other non-HLA antibodies in kidney transplant patients.

Antigen	N	Study design	Samples
GBM isolates: including agrin, perlecan	32	Case-control	Posttransplant
AT_1R, collagen IV, fibronectin	48	Cohort	Pre- or posttransplant
Collagen IV, fibronectin	36	Case-control	Pre- and posttransplant
Endoglin, EDIL3, ICAM4, FLT3 ligand	160	Cohort	Pre- and posttransplant
LG3-domain of perlecan	60	Case-control	Pre- and posttransplant
TLX	100	Cohort	Pretransplant
GSTT1	46	Case-control	Posttransplant
GSTT1, MICA	58	Case-control	Pre- and posttransplant
H-Y	133	Cohort	Pre- and posttransplant
Micro-array including MIG, ITAC, IFN-γ, GDNF	98	Case-control	Pre- and posttransplant
Nucleolin	117	Case-control	Pre- or posttransplant
PECR, PPIA, AURKA	66	Case-control	Pre- and posttransplant
PKCζ	43	Case-control	Pre- and posttransplant
SPDYA	15	Cohort	Posttransplant
Vimentin	163	Case-control	Pre- or posttransplant
Vimentin	70	Case-control	Pre- and posttransplant
β2-glycoprotein I	269	Cohort	Pretransplant

Abbreviations: AMR, antibody-mediated rejection; AT_1R, angiotensin type 1 receptor; AURKA, serine threonine kinase 6; CAN, chronic allograft nephropathy; EDIL3, EGF-like repeats and discoidin I-like domains 3; FLT3 ligand, Fms-related tyrosine kinase 3 ligand; GBM, glomerular basement; GDNF, glial derived neurotrophic factor; GSTT1, Glutathione S-transferase T1; HLA, human leukocyte antigen; ICAM4, intercellular adhesion molecule 4; IFN-γ,

Main outcome	Author
Significant higher incidence of antibodies in patients with transplant glomerulopathy compared with patients with CAN; higher number of rejection episodes.	Joosten, 2005 [104]
No significant association with acute rejection and graft function.	Hesemann, 2015 [19]
De novo antibodies strongly associated with transplant glomerulopathy.	Angaswamy, 2014 [105]
Antibodies against ≥1 antigens associated with AMR; patients with antibodies against all 4 antigens had significant more transplant glomerulopathy compared with patients without antibodies.	Jackson, 2015 [17]
Pre- and posttransplant anti-LG3 levels associated with acute vascular rejection.	Cardinal, 2013 [37]
Improved graft survival.	Padányi, 1996 [106]
Higher incidence in patients with (acute) rejection.	Akgul, 2012 [107]
Posttransplant anti-GSTT1 antibodies associated with C4d positive AMR.	Álvarez-Márquiz, 2009 [108]
De novo antibodies associated with acute rejection.	Tan, 2008 [109]
Pretransplant antibodies against MIG, ITAC, IFN-γ, GDNF correlated with CAN; >30 *de novo* non-HLA antibodies correlated with CAN severity.	Sigdel, 2012 [110]
Higher incidence of anti-nucleolin antibodies in patients with irreversible rejection compared with patients on the waiting list.	Qin, 2011 [111]
Only anti-PECR immunity was associated with transplant glomerulopathy.	Dinavahi, 2011 [54]
High levels at time of acute rejection associated with lower graft survival.	Sutherland, 2009 [112]
Antibody titers correlated with decline in graft function.	Li, 2010 [113]
Higher anti-vimentin antibody levels in patients with a failed allograft compared with non-transplanted patients with end-stage renal disease.	Carter, 2005 [53]
Higher mean anti-vimentin IgG levels posttransplantation in patients with IFTA.	Besarani, 2014 [27]
Higher incidence of early graft loss and delayed graft function.	Morales, 2014 [114]

interferon γ; ITAC, Chemokine (C-X-C motif) ligand 11; MIG, Chemokine (C-X-C motif) ligand 9; n, number of patients; PECR, peroxisomal-trans-2-enoyl-coA-reductase; PKCζ, protein kinase C ζ; PPIA, peptidyl-prolyl-isomerase-A; TLX, trophoblast-lymphocyte cross-reactive antigen; SPDYA, speedy homologue A.

Sensitization against non-HLA antigens in renal transplantation

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Abstract

Introduction

Donor-specific anti-HLA antibodies are a well-known risk marker for antibody-mediated rejection and graft loss following kidney transplantation. Circumstantial data suggests that non-HLA antibodies might also contribute to acute rejection and graft failure. Non-HLA antibody formation could be the result of graft damage and inflammation. We hypothesized that ischemia reperfusion injury at time of transplantation and rejection may lead to sensitization against non-HLA antigens. In this explorative study we assessed pretransplant sensitization against non-HLA in patients who developed rejection as compared with patients who did not. In addition, we studied whether sensitization posttransplantation occurs and whether this was related to occurrence of acute rejection within the first year.

Methods

We used an in house developed multiplex assay to measure sensitization against 12 different non-HLA antigens in serum samples of 99 transplant samples that were taken pretransplantation and at month 1, 3, 6 and 12 posttransplantation.

Results

In patients developing acute rejection within the first year we observed higher pretransplantation reactivity against ET_AR compared with patients who did not develop acute rejection (p=0.049). In addition, these patients show a tendency towards a higher reactivity against ARGHDIB pretransplantation (p=0.09). For the other 10 non-HLA antigens, no differences in pretransplant reactivity were observed. There was no significant increase in reactivity against the investigated non-HLA antigens over time posttransplantation compared with pretransplantation, both in patients with and without rejection.

Conclusion

In conclusion, these data suggest that pretransplant sensitization against ET_AR and ARGHDIB may be associated with acute rejection following transplantation. Moreover, transplantation and rejection, under immunosuppression, did not lead to direct sensitization against the investigated non-HLA antigens.

Introduction

Donor-specific anti-HLA antibodies (DSA) are a well-known risk marker for antibody-mediated rejection and graft loss following kidney transplantation [1]. However, not in all cases of antibody-mediated rejection DSA can be detected [2,3]. Moreover, in HLA-identical sibling transplantations peak panel reactive antibody reactivity strongly correlates with graft survival, suggesting that non-HLA antibodies may also be involved [4]. Numerous non-HLA antibodies, directed against allo- and autoantigens, have been identified thus far [5,6]. Potential mechanisms by which non-HLA antibodies may induce injury to the allograft include complement activation and antibody-dependent cell-mediated cytotoxicity, but also altered cell signaling and mimicry of receptor activation by hormones [7]. Most of the identified non-HLA antigens are ubiquitously expressed throughout the body and non-HLA antibodies also frequently occur within the healthy population [6,7]. This could indicate that non-HLA antibodies require changes in the microenvironment such as ischemia-reperfusion injury or binding of DSA to HLA on the donor endothelium in order to mediate graft injury [8].

There appears to be no association between non-HLA antibody positivity and classical sensitizing events (blood transfusion, pregnancies or previous transplantation) [5,6]. Non-HLA antibody formation may be the result of graft damage and inflammation leading to expression of intracellular proteins on apoptotic cells, shedding or cleavage of extracellular domains of surface-bound proteins or the release of donor-derived extracellular vesicles [5,8]. Sensitization against non-HLA antigens could also be due to antigen mimicry between viruses and non-HLA antigens, resulting in cross-reactivity [5]. Based on the potential triggers for non-HLA antibody formation, we hypothesize that *de novo* non-HLA antibodies may arise following transplantation. Limited information on the development of *de novo* non-HLA antibodies posttransplantation is available. A study on anti-endothelial cell antibodies (AECA) indicated that 10% of kidney transplant recipients developed *de novo* AECA posttransplantation and over half of these patients became positive for AECA within the first three months [9]. For AT_1R, two independent studies reported that the incidence of *de novo* antibodies within five years posttransplantation is around 3% [10,11].

Most studies thus far focus only on a single or a few non-HLA antibodies. The luminex multiplex technique enables to test for the presence of antibodies against multiple antigens at the same time [12]. Our lab has recently developed a non-HLA luminex assay that includes multiple non-HLA antigens that were identified in patients with end-stage renal disease or following kidney transplantation [13]. The aim of this explorative study was to generate new hypotheses on potentially relevant non-HLA targets and sensitizing events.

First, we looked whether there was a difference in pretransplant sensitization against non-HLA antigens between patients who developed acute rejection within the first year and those who did not. Secondly, we investigated whether sensitization following kidney transplantation or rejection occurred.

Methods

Patients

Ninety-nine patients who received a kidney transplant in the University Medical Center Utrecht between May 2015 and June 2017 were included in this study. All patients provided written informed consent to collect clinical data and serum samples pretransplantation and at month 1, 3, 6 and 12 posttransplantation. In case of graft failure, no further serum samples were collected. Pretransplant anti-HLA antibody status was determined in all patients as routine care with a single antigen bead assay (LABScreen PRA and Single Antigen class I and II, One Lambda, Canogan Park, CA) using a median fluorescence intensity (MFI) cut-off ≥500. No routine posttransplant HLA antibody monitoring was performed. Standard initial immunosuppressive therapy consisted of tacrolimus, mycophenolate mofetil and prednisolone. Induction therapy with an Interleukine-2 receptor monoclonal antibody (MoAb) or anti-CD52 MoAb was only given to patients at immunological increased risk. All kidney biopsies were performed on indication (rise in serum creatinine) and evaluated by an experienced nephropathologist.

Non-HLA antibody screening

We used an in house developed luminex assay, that was developed as part of the PROCARE consortium study, to test for antibody reactivity against 12 non-HLA targets [14]. The development and validation of this assay has been described in detail elsewhere [13]. For the purpose of this study we only included non-HLA antigens with a HaloTag that were coupled with a HaloTag amine ligand to a MagPlex microsphere (Luminex, Austin, Tx). The HaloTag enables complete 3d-exposure of the antigen, whereas in case of direct coupling of antigens to a bead not all epitopes are accessible for antibody binding. The assay also includes a positive control bead coated with human IgG and two negative controls beads: an empty and a transferrin coated bead. We measured all available serum samples with the same bead mixture on the same day on a LABScan 100 flow analyzer (OneLambda). For each non-HLA antigen we calculated the signal-to-background ratio (STBR) by dividing the MFI of the specific bead by the MFI of the transferrin-coated bead as the transferrin signal correlates best with the specific signal [13]. No cut-off was applied to define non-HLA antibody positivity.

Statistical analysis

We tested for overall differences in STBRs between groups with a Mann-Whitney U Test. To determine whether the STBR increased following transplantation, we compared the posttransplant ratios with the pretransplantation measurement with the Wilcoxon signed rank test. Data was analyzed with graphpad prism 7 (Graphpad Software Inc., Sa Diego, CA).

Results

We classified patients into three groups: primary nonfunction, acute rejection within the first year or stable graft function without rejection. Causes for primary nonfunction were renal vein thrombosis (n=2), arterial thrombosis (n=1) and a bleeding (n=1). Fifteen patients suffered from one or more episodes of biopsy proven acute rejection, these episodes were classified as T-cell mediated rejection (n=7), antibody-mediated rejection (n=6) or combined T-cell and antibody-mediated rejection (n=5). The median time to first rejection was 12 days (IQR 6-45). Baseline characteristics for the three groups are provided in table 1. Patients without rejection were less sensitized, cold ischemia time in deceased donor transplantation was shorter and donors were younger.

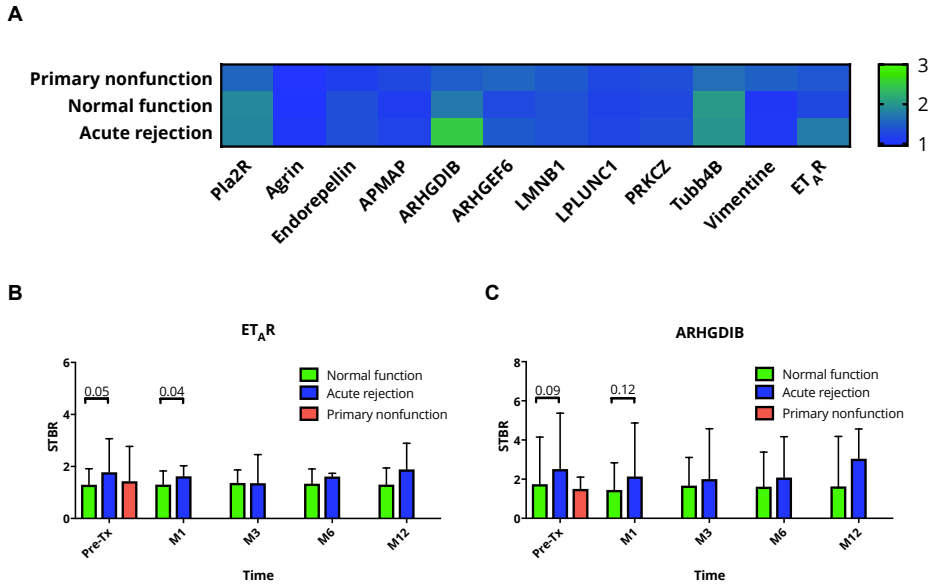
Table 1. Baseline characteristics.

	Primary nonfunction (n=4)	Rejection (n=15)	No rejection (n=80)
Donor age	62 (57-64)	62 (57-65)*	54 (47-60)
Donor sex, female	2 (50%)	8 (50%)	47 (59%)
Donor type:			
• Living	2 (50%)	7 (47%)	46 (58%)
• Donation after brain death	1 (25%)	1 (7%)	13 (16%)
• Donation after circulatory death	1 (25%)	7 (47%)	21 (26%)
Recipient age	49 (42-53)	53 (42-58)	55 (45-64)
Recipient sex, female	3 (75%)	6 (40%)	24 (30%)
Pre-emptive	0	6 (40%)	24 (30%)
Retransplantation	1 (25%)	4 (27%)	12 (15%)
Pregnancies	1/3	5/6	16/24
History of blood transfusions	2 (50%)	6 (40%)	24 (30%)
Pretransplant HLA antibodies	2 (50%)	10 (68%)*	24 (30%)
Pretransplant DSA antibodies	1 (25%)	5 (33%)*	10 (13%)
Peak panel reactive antibodies	14 (0-43)	0 (0-16)	0 (0-0)
Cold ischemic period[±]	16 (13-19)	23 (20-24)*	14 (12-19)
Delayed graft function	-	6 (40%)*	10 (13%)
Induction therapy	3 (75%)	10 (68%)	33 (41%)

[±]For deceased donors only. *Compared with patients without rejection $p<0.05$.

First, we compared the median non-HLA-STBRs at time of transplantation for the different groups (figure 1A). The patients in the rejection group showed a higher pretransplant endothelin type A receptor (ET_AR)-STBR compared with the group with normal graft function (p=0.049; figure 1B). In addition, there was tendency towards a higher median pretransplant ARHGDIB-STBR (p=0.09; figure 1C). For the other non-HLA antigens, no dissimilarities were observed. There were no significant differences between patients with TCMR and patients with AMR or mixed rejection (data not shown). Lastly, there were also no significant dissimilarities between the patients with primary nonfunction and the patients with stable graft function or rejection.

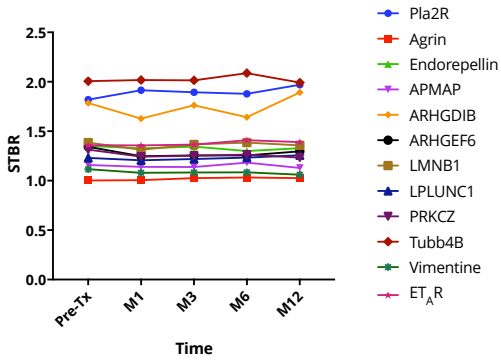
Figure 1. Signal-to-background ratio (STBR) against the different non-HLA antigens.



(A) Heat map showing median pretransplant STBR against non-HLA antigens. (B) ET_AR-STBR was significantly increased in patients who suffered from acute rejection in the first year compared with patients in the normal function group pretransplantation and at month 1. (C) There was a trend towards a higher ARGHDIB-STBR in the acute rejection group compared with the normal function group pretransplantation and at month 1. Data are depicted as median and interquartile range.

To assess whether transplant patients, irrespective of whether they develop rejection or not, become sensitized against non-HLA antigens following transplantation, we compared the posttransplant STBRs with the pretransplant STBR. Patient with primary nonfunction were not included in this analysis. As depicted in figure 2, there were no significant changes in the different non-HLA-STBRs over time. Next, we performed separate analyses on patients receiving a kidney from a deceased donor and on patients with rejection; also within these groups no significant changes were observed over time (data not shown).

Figure 2. Signal-to-background ratio (STBR) over time.



Median STBR against non-HLA antigens over time posttransplantation (months). No significant increase in STBR against any of the non-HLA antigens occurred over time. Patients with primary nonfunction were excluded from this analysis. Data were analyzed with the Wilcoxon matched-pairs signed rank test.

Finally, we looked at non-HLA-STBRs at time of acute rejection and compared these to all posttransplant measurements in the patients without rejection. The median ET_AR-STBR was significantly higher at time of acute rejection compared with the median posttransplant ratio in patients with no rejection (1.67 vs. 1.33, p=0.02). For ARGHDIB and the other non-HLA antigens, no significant differences were observed.

Discussion

This explorative study was set out to generate new hypotheses about the potential association between non-HLA antibodies and acute rejection and sensitizing events leading to antibody development. The results indicate that patients who developed acute rejection within the first year showed pretransplantation a higher reactivity against ET_AR compared with patients who did not develop acute rejection. In addition, they showed a tendency towards a higher reactivity against ARGHDIB pretransplantation. Based on our findings, we suggest that future studies on the impact of non-HLA antibodies on acute rejection should include these targets as part of their non-HLA antigen panel. There was no significant increase in reactivity against the investigated non-HLA antigens over time posttransplantation compared with pretransplantation. This indicates that other mechanisms involved in sensitization against non-HLA antigens should be considered.

The 12 non-HLA targets included in this assay were selected based on their description in kidney transplant patients or patients with end-stage renal disease [13]. The median ET_AR-STBR was increased at time of acute rejection as well as pretransplantation and

at month 1 compared with patients who did not develop acute rejection within the first year. ET_AR is one of the receptors for endothelin and is expressed amongst others on vascular smooth muscle cells. Activation of ET_AR by endothelin leads to vasoconstriction and has pro-inflammatory effects including cytokine production and macrophage infiltration [15,16]. Anti-ET_AR antibodies may mimic endothelin-induced receptor activation [7,15]. In line with our observations, pretransplant anti-ET_AR antibodies have been associated with poorer graft function at one year posttransplantation and with more severe vascular rejection [15]. Patients with acute rejection also showed a tendency towards a higher median ARGHDIB (Rho GDP dissociation inhibitor 2)-STBR pretransplantation and at month 1 compared with the group without rejection. Reactivity against ARGHDIB was frequently observed in a group of hemodialysis patients [17], but has not been related to transplant outcomes before. Proteins of the Rho family are involved in multiple cellular events including cell proliferation and signaling and are only activated in bound-state [18,19]. ARGHDIB decreases the dissociation rate of Rho family proteins and is considered to be involved in multiple malignancies [18,19]. We did not observe any difference in pretransplant sensitization against the other 10 non-HLA targets between patients with and without rejection in the first year. Of these targets, only endorepellin, LPLUNC, APMAP and PRKCZ have been studied in relation to acute rejection [20-22]. Cardinal et al. observed that anti-endorepellin levels were increased in acute vascular rejection, but not in acute tubulo-interstitial rejection [22]. In addition, passive anti-endorepellin transfer in a mouse aortic transplantation model led to increased C4 deposition, neointima formation and allograft inflammation [22]. In our study, endorepellin-STBR was not significantly increased at time of rejection or for any of time points in the rejection group. Potentially, we did not identify endorepellin as a target because of the low prevalence of pure vascular rejection (n=4). Reactivity against LPLUNC, APMAP and PRKCZ was described in small case-series and has not been further evaluated [20,21]. The other non-HLA antibody targets were identified in patients awaiting transplantation [17] or chronic renal allograft nephropathy [23-25] and may not be related to acute rejection. In this study we could not look at reactivity against the AT_1R, one of the most studied non-HLA target in kidney transplant recipients thus far [10,26-28], as this target was not available with a HaloTag coupling in our assay. Although several studies showed that AT_1R antibodies were associated with rejection and graft survival, a recent large confirmation study (n=940) did not confirm this association [28]. Reactivity patterns against multiple non-HLA antigens may eventually provide more information compared with just a single non-HLA antigen.

In this explorative study we compared STBRs and did not apply a cut-off to define non-HLA antibody positivity. Given the frequent occurrence of non-HLA antibodies within the healthy population [6,7], ideally a clinically relevant cut-off should be applied. Because of the limited sample size we could not determine such cut-off values. Within the PROCARE

cohort, clinically relevant cut-offs for this non-HLA were identified based on differences in long-term graft survival [13]. As the prevalence of the different non-HLA antibodies based on these cut-off was low (<5%) and we did not look at graft survival in this study, we decided to not apply these cut-offs in the present study.

The small sample size in combination with relatively large interindividual variations could have led to lack of significant differences between groups but also to a falsely significant result due to random sampling error. We did not adjust for multiple testing, as the purpose of this study was to generate new hypotheses. Another limitation is that we only had information on pretransplant DSA and not on *de novo* DSA development. Taniguchi et al. looked at *de novo* AT_1R-antibody and DSA development posttransplantation and identified several patterns including the synergetic occurrence of both antibodies [10]. We observed within the first year posttransplantation no significant increase in STBR for any of the non-HLA antigens, also not when we only looked at patients who received a kidney from a deceased donor who have more ischemia-reperfusion injury compared with patients receiving a kidney from a living donor [29]. This suggests that no distinct direct sensitization following transplantation does occur. In line with this, Gareau et al. recently applied a new non-HLA multiplex luminex assay including 63 targets on serial samples from a selected population of 101 patients [26]. Although they did not specifically tested changes over time, their data suggest no distinct changes in median MFI at month 6 and 24 posttransplantation compared with pretransplantation. Moreover, Wiebe et al. showed that *de novo* DSA develop on average at 4.6 (± 3.0) years posttransplantation, with none of the patients developing *de novo* DSA within the first six months [30]. The development of *de novo* DSA has been strongly associated with inadequate immunosuppression and particularly non-adherence [30,31]. Possibly the usage of induction and triple therapy at time of transplantation might prevent against direct sensitization to non-HLA antigens. It would be interesting to re-evaluate all patients in a few years to see whether sensitization occurs later on.

In conclusion, this study opens new perspectives on the role of ARGHDIB and ET_AR in acute renal allograft rejection. In addition, no distinct sensitization against the investigated non-HLA antigens occurred within the first year posttransplantation. Future research might focus on clinical relevant cut-offs for non-HLA antibodies related to the occurrence of acute rejection and *de novo* development of these antibodies in the long-term.

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PART 2

Complement regulation

Complement polymorphisms in kidney transplantation: critical in graft rejection?

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Abstract

The complement system, as part of the innate immune system, plays an important role in renal transplantation. Complement is involved in the protection against foreign organisms and clearance of apoptotic cells but can also cause injury to the renal allograft for instance via antibody binding or in ischemia reperfusion injury. Numerous polymorphisms in complement factors have been identified thus far; some of them result in different functionalities or alter complement levels. In this review, we provide an overview of the literature on the role of complement polymorphisms in renal transplantation. Furthermore, we discuss functional complement polymorphisms that have not yet been investigated in kidney transplantation. By investigating multiple polymorphisms both in donor and recipient at the same time, a complotype can be constructed. Because the combination of multiple polymorphisms is likely to have a greater impact than a single one, this could provide valuable prognostic information.

Introduction

The innate immune system is an important first line defense barrier against foreign organisms. It is increasingly being emphasized that innate immunity plays an important role in the initiation of renal transplant rejection [1]. Complement, one of the components of the innate immune system, is not exclusively involved in rejection but also in other complications including ischemia reperfusion injury (IRI), infections and recurrence of some primary renal diseases [2,3]. The complement system constitutes of over thirty proteins, both circulating in plasma and membrane-bound [1]. Activation of the complement cascade via the classical, lectin or alternative pathway will result in the cleavage of C3 and C5 and eventually assembling of C5b with C6, C7, C8 and C9 to form the membrane-attack complex. Complement regulators, both fluid-phase and membrane-bound, control amplification of the complement cascade and protect (self-)cells against the deleterious effects of complement activation [4]. Numerous polymorphisms in complement proteins have been identified thus far, including several polymorphisms that result in distinct functional differences (Figure 1) [5-8]. For instance, serum mannose-binding lectin (MBL) levels are largely influenced by polymorphisms both in the promotor and the coding part of the (MBL2) gene [9].

A complete summary of the polymorphisms studied in renal transplantation is provided in Table 1. Genetic differences in genes coding for complement (regulatory) components could influence the level of inflammatory responses, thereby influencing susceptibility to acute rejection and allograft loss.

C3

C3 polymorphisms may be particularly relevant in complement mediated allograft damage as C3 is a central factor in the complement cascade. Although C3 is mainly produced in the liver, it is also synthesized in other tissues including the kidney. Donor-derived C3 that is primarily produced by tubular epithelial cells, contributes to 5% of the total circulating C3 pool in steady state and this may increase to 16% at time of acute rejection [25]. Animal studies indicated that in the absence of local produced C3, transplanted mice show much less vigorous signs of IRI and rejection [26,27]. Therefore, C3 polymorphisms in both donor and recipients may alter complement-mediated allograft damage. Based on the electrophoretic motility two common C3 allotypes are distinguished: C3S (slow) and C3F (fast). These allotypes are identified by a single nucleotide polymorphism (SNP) resulting in the replacement of arginine (C3S) for glycine (C3F) [28]. This arginine nucleotide conformation is essential for the stabilization of C3b-factor H complexes that accelerate the decay of C3 convertase, thereby inhibiting the amplification of C3 [7]. The C3S allele is the predominant form and is present in 80% of Caucasians, 95% among blacks and 99% among Asian [13].

Table 1. Summary of studied complement polymorphisms in renal transplantation.

Complement protein	SNP - rs number	Location	Functional effect
C3	rs2230199	Exon	Less efficient binding of C3Fast variant to factor H.
C4A and C4B*	Insertion of 6.36kb endogenous virus HERV-K in C4 long variant.	Intron	Lower C4 levels probably due to epistatic effects.
C5	rs17611	Exon	Increased C5 turnover.
C5a receptor	rs10404456	Intron	No direct, haplotype tagging SNP.
	rs4804049	Exon	None.
Ficolin-2	rs3124952	Promoter	Affect serum ficolin-2 levels.
	rs3124953	Promoter	
	rs17514136	5′UTR	
	rs7851696	Exon	
	rs17549193	Exon	
MASP2	rs56392418	Exon	Affect serum MASP2 levels.
	rs12085877	Exon	
	rs1033638	3′ UTR	
	rs72550870	Exon	
MBL	rs1800450	Exon	Reduced formation of high-order oligomers and subsequent lower MBL plasma levels.
	rs1800451	Exon	
	rs5030737	Exon	
	rs7095891	Promoter	Influence transcriptional activity.
	rs7096206	5′UTR	
	rs11003125	Promoter	
CD46	rs2796267	Promoter	No direct, part of a haplotype that is associated with lower transcription activity.

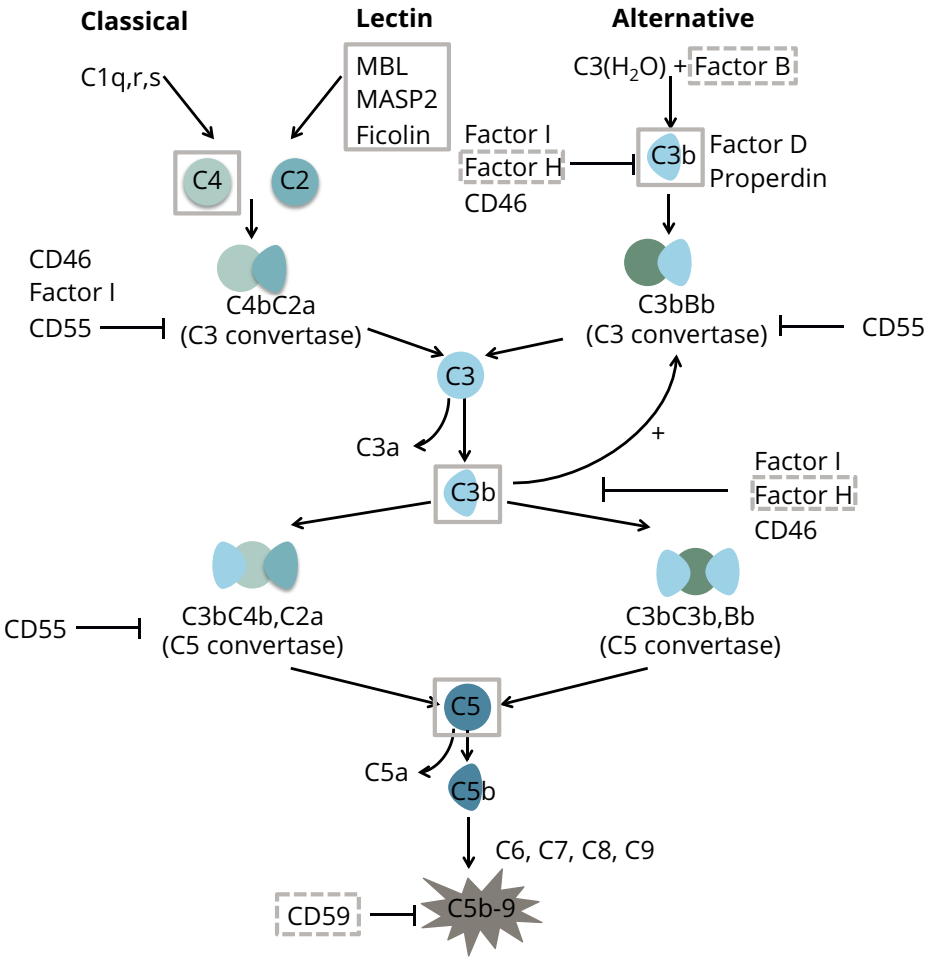
* C4A and C4B share >99% sequence identities. Total gene copy numbers may vary from 0-8 and both genes can be present as a short or long variant.

Six other haplotype tagging non-functional SNPs in the C5 gene and one in the C5a receptor gene were also genotyped in this study.

Abbreviations: MASP2, mannan-binding lectin-associated serine protease 2; MBL, mannose-binding lectin; SNP, single nucleotide polymorphism; UTR, untranslated region.

	Clinical relevance in kidney transplantation	References
	No distinct evidence of an association, both for donor and recipient allotype, with transplant outcomes.	[10-14]
	Based on available studies, insufficient evidence for clinical relevance in kidney transplantation.	[15,16]
	Part of linkage disequilibrium block that has been associated with graft function on year 1 and 7, not graft survival. Warrants validation.	[17]#
	No association with transplant outcomes reported.	[17]#
	Associated with graft failure in a case-control study. Warrants validation.	[18]
	No association with graft survival and acute rejection reported.	[19,20] [19]
	No association with graft survival and acute rejection reported.	[20] [19]
	No overall effect. Low-expressing MBL-genotypes may be associated with impaired graft survival and incidence of acute rejection in non-HLA immunized patients or recipients with a deceased donor.	[19-23] [19-21,23] [19-21,23] [19-21,23] [19] [19-21]
	Associated with acute and late rejection, not graft survival. Warrants validation.	[24]

Figure 1. Functional polymorphisms in complement factors and regulating proteins.



An uninterrupted line highlights complement proteins with known functional polymorphisms that have been studied in renal transplantation and a dotted line highlights those that have been investigated in other conditions.

The role of C3 allotype in renal transplantation was first studied by Andrews et al., who showed that the presence of a C3F allele in donor or recipient was associated with a higher incidence of graft dysfunction, but not graft survival or number of rejection episodes [10]. In contrast, Brown et al. found that recipients of a C3F/S or C3F/F donor showed significantly improved graft function and graft survival from one year onwards. Subgroup analyses revealed that the observed effect on graft survival was even stronger amongst deceased donors, whilst no significant effect was seen in living donors [11]. However, subsequent studies could not confirm these findings (Table 2) [12-14]. Though, *post hoc*

subgroup analyses in the study by Damman et al. revealed that recipients of donation after cardiac death (DCD) donors with a C3F allotype (n=201) had a markedly lower risk of primary nonfunction compared with recipients of C3S/S DCD donors (adjusted HR 0.12 (0.02–0.73)) [13].

The studies on C3 allotype highlight the importance of validation studies with adequate sample sizes following initial discovery studies. Two independent validation studies by Varagunam et al. and Damman et al. including each more donor-recipient pairs than the three other studies combined [10,11,14] and with longer mean follow-up times, could not find any association between C3 allotype of donor nor recipients and graft survival or acute rejection. Therefore, it is unlikely that C3 allotype influences long-term grafts survival. The finding by Damman et al. on C3 allotype and incidence of primary nonfunction requires further validation. A potential protective effect of C3F seems in contrast to the adverse impact on other complement-mediated diseases such as age-related macular degeneration (AMD) and IgA nephropathy and the less efficient binding of C3F to factor H resulting in more activation of the alternative pathway [7,28]. However, it could be that increased C3 levels contribute to more effective clearing of apoptotic cells following IRI by mechanisms independent of downstream complement activation, resulting in lowered incidence of primary nonfunction [29].

Table 2. Clinical studies on C3 Fast and Slow variant in kidney transplantation.

Cohort	N (donor + recipient pairs)	Donor type	Key findings
Andrews, 1995 [10]	183	Deceased	Presence of C3F allele in donor or recipient was associated with allograft dysfunction, not graft survival.
Brown, 2006 [11]	662	Deceased + living	Recipients of a C3F/S or C3F/F donor showed improved graft survival.
Varagunam, 2009 [12]	1147	Deceased	No association with renal allograft outcome.
Damman, 2012 [13]	1265	Deceased + living	No overall association with renal allograft outcome. Kidneys from C3F donation after cardiac death donors showed lower incidence of primary nonfunction.
Bazyar, 2012 [14]	100	Mainly living	No association with acute rejection.

C4

C4, like C3, is mainly produced by the liver but can also be locally synthesized by tubular epithelial cells [30]. There are two C4 isotopes C4A (acidic) and C4B (basic) that are encoded by the C4A and C4B genes and share >99% sequence identities. C4A is primarily involved in the clearance of immune complexes through its affinity for amino groups, whereas C4B favorably binds to hydroxyl groups to form an ester linkage with carbohydrate structures [31]. Most individuals carry two C4A genes and two C4B genes, however, the total number of genes may vary from zero to eight [31]. Gene copy numbers are strongly related to C4 plasma levels [31]. Furthermore, there is also a short and a long version of both C4 genes (C4S and C4L) [15]. The long variant is associated with lower C4 plasma levels, this is probably a result of the negative epistatic effects of an endogenous retrovirus present[31]. Since C4 is centrally involved in the formation of C3 convertase in the classical and lectin pathway, plasma levels may contribute to the degree of further downstream amplification and thereby influence the severity of complement-mediated damage in the allograft upon ischemia reperfusion and antibody binding.

Wahrman et al. evaluated 1946 deceased donors and their corresponding recipients for C4A and C4B copy numbers and C4 short and long variants. The C4 copy number, both in donor and recipient, did not correlate with 10-year graft survival, patient survival, rejection treatment, graft dysfunction or infectious episodes. The only significant association with impaired graft survival was seen in recipients with ≥1 C4S copies that received a kidney from a null C4S donor (HR 0.73, $p = 0.016$) [15]. In contrast, Bay et al. found in recipients from a deceased donor (n=456) that a C4 copy number <4 conferred a lower risk of 5-year allograft failure (HR 0.43, $p = 0.021$) but not 10-year allograft failure. A similar trend could not be observed in recipients of a living donor (n=220). The authors hypothesize that because a deceased donor kidney suffers from prolonged ischemia, it may be more prone to antibody binding and complement activation and therefore differences in C4 copy number only affect graft survival of a deceased donor kidney[16]. However, the sample size of this latter cohort is too small considering the multiple hypotheses that were tested. Besides, recipients from a deceased donor with a C4 copy number <4 tended to have a slightly lower mean number of HLA A and B mismatches (p=0.05) compared with recipients of donor with >4 C4 copy numbers [16]. Since no adjusted analyses were reported, this may have attributed to improved graft survival in recipients of a deceased donor with <4 C4 copy numbers. Therefore, based on available studies there is insufficient evidence supporting an association between C4 copy number and transplant outcomes.

C5

The C5 gene is highly polymorphic [17]. One of the identified SNPs rs17611, a missense polymorphism, is associated with an increased C5 turnover resulting in a significant decrease of plasma C5 and increase in C5a levels [6]. This could in turn enhance chemoattraction and potentially also membrane attack complex formation resulting in more severe graft injury. The anaphylatoxin C5a exercises its role via the C5a receptor (C5aR) that is expressed amongst others on neutrophils and activated B and T cells [18]. Polymorphisms in the C5aR gene could therefore influence T-cell mediated injury to the graft.

Jeong et al. looked at C5 and C5aR gene polymorphisms trough haplotype tagging SNPs, including rs17611, in a population of 191 Korean donor-recipient pairs. The other 7 SNPs that were genotyped were all intronic or synonymous. After correcting for multiple testing, none of the individual SNPs was associated with renal function or grafts survival. However, a certain haplotype configuration for one of the haplotype blocks, both in recipient and donor, was associated with a lower eGFR at 1 and 7 years posttransplantation. There was no significant association between this haplotype configuration and graft survival. The authors argue that this could be because of a relatively short follow-up period and low number of allograft loss (n=15) [17]. Based on this small study, no conclusions on the potential association between C5 and C5aR polymorphisms and renal transplantation can be drawn.

Mannose-binding lectin pathway polymorphisms

Serum MBL concentrations are largely influenced by genetic polymorphisms in the MBL2 gene. The most important polymorphisms affecting serum levels are three variant alleles in exon1 and two promoter SNPs affecting transcriptional activity (Table 1) [9,19]. Based on the serum MBL levels, genotype combinations are usually merged into a high and low expressing genotype [9]. Similarly, serum ficolin-2 (FCN2) and MBL-associated serine protease 2 (MASP2) concentrations are largely affected by polymorphisms in their coding genes [32,33]. It is hypothesized that MBL functions as a double-edged sword in transplant immunity [20,34]. Severe tissue injury may result in exposure and subsequent binding of both low- and high-order MBL to tubular epithelial cells resulting in complement-independent cell cytotoxicity and enhanced alloantigen presentation [34,35]. In the absence of a strong inflammatory response, MBL could have a protective effect by an augmented clearance of apoptotic allograft cells and thereby dampen the alloimmune response [20,34].

Serum MBL concentrations in renal transplant patients were first studied by Berger et al. in 266 recipients of deceased donors, indicating that high MBL levels (>400ng/ml) were associated with impaired 10-year death-censored graft survival (relative risk 2.76, 95%CI 1.2-6.32) [36]. In contrast, Damman et al. and Bay et al. showed no overall effect of MBL levels or genotype on graft survival but found in recipients of a deceased donor or in the absence of pretransplant HLA-immunization against the graft, an unfavorable effect of a low-expressing MBL genotype on graft survival (Table 3) [19,23,34]. Moreover, other studies looking at different outcomes suggested that low-expressing MBL genotypes may predispose to acute rejection [20,22] or cytomegalovirus disease [21]. Damman et al. and Golshayan et al. also looked at FCN2 and MASP2 genotypes but found no association with graft survival and rejection [19,20].

Table 3. Clinical studies on mannose-binding lectin (MBL) polymorphisms in kidney transplantation.

Cohort	N (recipients)	Donor type	Key findings
Berger, 2005 [36]	266	Deceased	High MBL levels were associated with an impaired graft survival.
Cervera, 2007[21]	236	Deceased + living	A trend towards a higher incidence of CMV pp65 antigenemia and CMV disease in patients with a low-expressing MBL genotype.
Gorgi, 2009 [22]	133	Not described	A tendency towards a higher incidence of acute rejection in patients with low-expressing MBL genotypes.
Damman, 2012 [19]	1271 donor-recipient pairs	Deceased + living	No overall effect on graft survival. Recipients of a fully HLA-matched graft with a low-expressing MBL genotypes showed impaired graft survival.
Bay, 2013 [23]	544	Deceased + living	No overall effect on graft survival. In non-HLA-immunized patients and recipients of a deceased donor, low-expressing MBL genotypes showed impaired graft survival.
Golshayan, 2016 [20]	710	Deceased + living	Low- or deficient MBL genotypes were associated with a higher incidence of acute cellular rejection in recipients of a deceased donor.

In conclusion, these studies indicate that MBL polymorphisms influence transplant outcomes in some patients. However, the studies do not fully agree on which transplantations are affected by MBL polymorphisms and whether high MBL levels are protective or harmful [19,20,22,23,34,36]. Damman et al. suggest that because Berger et al. used a technique to measure MBL levels that detects both low- and high-order MBL oligomers, their association between high MBL levels and impaired graft survival may be complement-independent since only high-order MBL oligomers can interact with carbohydrate

structures [34]. Another difficulty in comparing these studies is that not all studies included the same SNPs (Table 1) and there were small differences in the definitions of low and high expressing genotypes. Although this latter effect is probably limited, we suggest that future validation studies should preferably use the same study protocol as the largest study thus far by Damman et al. [19].

Non-coding polymorphisms

The above-mentioned polymorphisms are all in coding regions of the genome, and thereby directly define the functionality of complement proteins transcribed. Non-coding polymorphisms do not directly influence functionality of the transcript, but rather influence gene transcription levels through altered binding affinity of transcription factors and activators in the case of promoter and enhancer polymorphisms, or via linkage disequilibrium (LD) in the case of polymorphisms in intronic regions [37].

Three studies on non-functional polymorphisms in renal transplantation have been published thus far. First, a case control study was performed to investigate the influence of a silent mutation within the C5aR gene [18]. Recipients of a deceased donor who rejected their graft within the first year (n=265) had more often a variant allele compared with patient who had stable graft function (n=290). However, it should be taken into account that this SNP was only present in 4.7% of the transplanted patients [18]. Because a minor allele frequency of 5% is associated with a higher change of finding false-positive associations compared with more frequent SNPs, these findings warrant further validation [38]. Second, Ermini et al. evaluated 1170 European donor-recipient pairs by a large multiplex polymerase chain reaction covering 505-tagged SNPs in 47 complement genes. Although they identified several non-coding SNPs in the lectin pathway and C7 with low p-values, these associations were not significant with graft survival after an extensive correction for multiple testing [39]. Lastly, a SNP in the promoter region of CD46 (rs2796267) was significantly associated with acute rejection and late acute rejection, but not with allograft loss in 334 kidney transplant patients [24]. These findings suggest that polymorphisms in (membrane bound) complement regulators may also affect transplant outcomes.

Potential other relevant functional complement polymorphisms

Functional complement polymorphisms have been studied in various other diseases as well. In this paragraph we will discuss a few functional polymorphisms that might also be relevant in renal transplantation in order to give an example of possibilities for future research.

Age related macular degeneration is the leading common cause of blindness in Western populations. It is believed that genetic variations in complement genes including C2, C3, factor B and H are involved in the susceptibility to AMD [40]. A functional SNP that is strongly associated with AMD is the Y402H coding variant in the factor H gene. This SNP is located in a region that binds to C-reactive protein and heparin and may therefore alter this binding, resulting in deregulation of factor H [8]. Polymorphisms in factor H may also be relevant in renal transplantation because of their critical role in maintaining balance between complement activation and inhibition on cell surfaces [4]. Other coding polymorphisms strongly associated with AMD are located in the C2 and complement factor B gene. The L9H variant in factor B and E318D variant in C2 are almost in complete LD and confer a protective effect (OR 0.37, 95%CI 0.18-0.60). Furthermore, the R32Q allele in factor B and rs547154 in C2 are also almost in complete LD and also confer a protective effect (OR 0.32, 95%CI 0.21-0.48). Because of the strong LD between the SNPs on C2 and factor B, it is not completely sure whether the protective effect should be attributed to factor B or C2 [41]. However, functional data is supporting a causative role for the R32Q allele. The fB_{32Q} variant appears to be less effective in amplification of the alternative pathway as a result of a lower binding-affinity of fB_{32Q} to C3b and subsequent lower levels of C3bBb convertase [5]. This may also be relevant in renal transplantation: less effective amplification of the alternative pathway could for instance protect against IRI.

Finally, in lung transplantation it was shown that a SNP in the promoter region of CD59 (rs147788946) in lung donors was associated with an increased risk for bronchiolitis obliterans syndrome (BOS) [42]. Functional experiments indicated that lung endothelial cells and monocytes derived from lung donors with this SNP configuration showed lower CD59 expression and were more susceptible to complement-mediated cell lysis [42]. None of the polymorphisms within the CD46 and CD55 promoters did correlate with incidence of BOS nor expression levels and susceptibility to complement-mediated cell lysis [42]. We hypothesize that recipients of kidney with this CD59 promoter SNP configuration may also be more prone to antibody-mediated rejection and impaired graft survival.

Concluding remarks

The complement system plays a pivotal role in innate immunity following kidney transplantation [3]. However, the balance between desirable effects such as protection against infections and the clearance of apoptotic cells and destructive complement-mediated inflammation following for instance antibody binding to the allograft is delicate [43]. Therefore, it is hypothesized that complement polymorphisms may influence transplant outcomes. Polymorphisms in MBL, C3 and C4 have been studied in multiple (mainly European) cohorts, though they do not show an indisputable association between functional complement polymorphisms and graft survival or acute rejection [10-16,19,20,22,23,34,36]. A major drawback of the available evidence is that validation studies are lacking or inadequately powered. Discovery cohorts are crucial to identify potential clinically relevant polymorphisms but need to be validated. Important aspects in designing these validation studies are a sample size calculation to obtain sufficient power, applying the same genotyping protocol and to have a follow-up time of at least five years when looking at graft survival. Preferably, the sample size would also enable to perform subgroup analyses for donor type and HLA-immunization. Functional complement polymorphisms may be less relevant in recipients of a living kidney donor as these transplantations are associated with less vigorous complement activation[44]. Furthermore, polymorphisms in the classical pathway or downstream in the cascade may primarily influence rejection rate and graft survival in patients with (non-)HLA antibodies, as these antibodies may evoke strong complement activation upon binding to the allograft [3].

The studies discussed in this review focus mainly on the effect of a single polymorphism instead of a combination of several polymorphisms in different complement factors. However, while individual polymorphisms may have only minor impact, interactions among different polymorphisms may have major impact on complement activity. This is supported by in vitro studies indicating that a combination of risk variants in C3, factor B and H results in a six-fold increase in complement activity [45]. The inherited set of polymorphisms in genes encoding for complement proteins and regulators is called a complotype and is believed to largely determine individual intrinsic complement activity [43]. Complotypes resulting in a more active complement system could make an individual more prone to inflammation, whereas combinations of polymorphisms resulting in a dampened complement system may put patients at risk for infection [43]. In AMD patients, it was recently shown that a combination of two SNPs in factor B and one in factor H conferred the highest association with disease status (p=5.84*10^{-13}) and complement activity in plasma in vivo (p=8.31*10^{-9}) [46].

Because the allograft is capable to synthesize C3, C4 and complement regulators, both membrane bound and fluid phase, donor-polymorphisms in these proteins may interact with polymorphisms in the recipient [3]. Therefore, constructing a complotype based on the complement genotype of both recipient and donor could provide a valuable risk stratification tool for acute rejection or allograft loss. In donor-recipient pairs with an active complotype, recipients may benefit from complement inhibitors such as eculizumab. Furthermore, this complotype may be taking into consideration along with other immunological risk factors to determine which patients could benefit from more aggressive immunosuppressive therapy. Future studies on the identification of this complotype should not only focus on complement factors but also on their regulating proteins including CD59, as these regulators are important in maintaining long-term allograft function for instance through their role in accommodation [1].

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Association between promoter polymorphisms in CD46 and CD59 in kidney donors and transplant outcome

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Abstract

Complement regulating proteins, including CD46, CD55 and CD59, protect cells against self-damage. Because of their expression on the donor endothelium, they are hypothesized to be involved in accommodation. Polymorphisms in their promoter regions may affect their expression. The aim of this study was to investigate if donor polymorphisms in complement regulating proteins influence kidney transplant outcomes. We included 306 kidney transplantations between 2005 and 2010. Five polymorphisms in the promoters of CD46, CD55 and CD59 were genotyped. A CD59 promoter polymorphism (rs147788946) in donors was associated with a lower one-year rejection-free survival (adjusted HR 2.18, 95%CI 1.12-4.24) and a trend towards impaired 5-year graft survival (p=0.08). Patients receiving a kidney with at least one G allele for the CD46 promoter polymorphism rs2796267 (A/G) showed a lower rejection-free survival, though this became borderline significant after adjustment for potential confounders (HR1.87, 95% CI 0.96-3.65). A second CD46 promoter polymorphism (rs2796268, A/G), was also associated with a lower freedom from acute rejection in the presence of at least one G allele (adjusted HR 1.95, 95% CI 1.03-3.68). Finally, the combined presence of both favorable genotypes of rs2796267 and rs147788946 had an additional protective effect both on acute rejection (p=0.006) and graft survival (p=0.03). These findings could help to identify patients who could benefit from intensified immunosuppressive therapy or novel complement inhibitory therapeutics.

Introduction

Current immunologic matching of donor and recipient and risk stratification in kidney transplantation is largely based on AB0 blood group compatibility, HLA typing and the presence of donor-specific HLA antibodies, but does not include the potency of effector mechanisms such as the complement system [1-3]. In kidney transplantation, complement activation can be involved in the pathogenesis of ischemia-reperfusion injury (IRI), both cellular and antibody-mediated rejection and posttransplant occurrence of certain native kidney diseases. Damage to the renal allograft is mainly mediated through the anaphylatoxins C3a and C5a, the opsonin C3b and the membrane attack complex, resulting in pore formation in the cell membrane and subsequent cell lysis [4]. To protect cells against complement-mediated damage, most nucleated cells express membrane bound complement regulatory proteins including membrane cofactor protein (CD46), decay accelerating factor (CD55) and protectin (CD59) [5]. CD46 is a cofactor for factor I and thereby facilitates degradation of the complement proteins C3b and C4b. Downstream amplification of the complement cascade is further inhibited by CD55 through the inhibition of C3 convertase. Lastly, CD59 interferes with the formation of the membrane attack complex by blocking the binding of C9 to C5b-C8 [5]. Animal and human-biopsy studies suggest that complement regulation by these proteins on the donor endothelium is crucial in accomplishing accommodation, inducing resistance against antibody-mediated complement-dependent cell lysis, and subsequent long-term allograft function [6-10]. A higher CD46 expression in renal tubules following treatment for acute T-cell mediated rejection was found to be associated with lower serum creatinine values and improved graft survival [7]. Moreover, another study reported that a diffuse positive CD55 staining in the peritubular capillaries of for cause renal allograft biopsies correlated with a smaller increase in serum creatinine and a better graft survival compared with biopsies with a negative CD55 staining [8]. Finally, large-animal studies demonstrated that rejected renal grafts show lower CD59 expression compared with accommodated grafts [9,10]. The importance of these complement regulators is further illustrated by their role in the pathogenesis of atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy, both complement deregulation disorders [11].

Multiple studies on complement polymorphisms in kidney transplantation, including in C3, C4 and mannose-binding lectin, have been performed, but could not provide an indisputable association between these polymorphisms and acute rejection nor graft survival [12]. We hypothesize that polymorphisms in genes encoding for membrane bound complement regulatory proteins in kidney donors may have a greater influence on acute rejection and graft survival because of their pivotal role in accommodation. Promoter polymorphisms can affect binding affinity of transcription factors and thereby protein

expression levels [13]. The genes encoding for CD46 and CD55 are located on chromosome 1 and for CD59 on chromosome 11. Within the CD46 promoter region, the GG haplotype of the single nucleotide polymorphisms (SNPs) rs2796267 and rs2796268 is associated with a lower transcriptional activity compared with the AA haplotype [14]. Rs2796268 is located within the consensus binding sequence of the transcription factor CBF-1/RBP-Jk [14]. Therefore, donor kidneys with at least one G allele for these SNPs may show a lower CD46 expression upon their endothelium compared with kidneys with a homozygous A genotype. Both G alleles are also part of larger aHUS risk haplotype [15]. A 21 base pair deletion in the promoter region of CD55 (rs150046210) is associated with a lower transcriptional activity and CD55 expression levels and with more severe influenza infections and allergic respiratory diseases [16,17]. Lastly, an adenine insertion in the promoter of CD59 (rs147788946) in lung donors is associated with the incidence of bronchiolitis obliterans syndrome (BOS) following lung transplantation [18]. The aim of this study was to investigate whether donor polymorphisms in the promoters of CD46, CD55 and CD59 affect kidney transplant outcomes.

Patients and methods

Patients and study design

Between January 2005 and December 2010, 357 transplantations were performed in the UMC Utrecht. 51 transplantations were excluded because no donor DNA was available (n=38) or donor DNA was degraded and not suitable for genotyping anymore (n=13), leaving 306 transplantations for this analysis. Clinical data was obtained from hospital records and the Dutch Organ Transplant Registry for which all patients provided written informed consent. Posttransplant follow-up data was available for all patients for at least 5 years. The primary outcomes in this study were 5-year death-censored graft survival and 1-year freedom from acute rejection. For acute rejection, we decided to look at 1-year freedom from acute rejection because the incidence of acute rejection is the highest within the first year and rejection after the first year is often related with nonadherence or overaggressive immunosuppressive tapering [19]. Rejection was defined as biopsy-proven acute borderline rejection or acute rejection. All biopsies were performed on indication and reviewed by an experienced nephropathologist according to the Banff classification valid at that time. The study protocol was approved by the Biobank Research Ethics Committee of the UMC Utrecht (TC Bio 13-633) and performed in accordance with the Declaration of Helsinki.

Immunological screening

In all transplantations the T-cell complement dependent cytotoxicity crossmatch with both current and peak sera was negative. In addition, pretransplant anti-HLA antibodies were previously determined with the LABScreen PRA and Single Antigen class I and II (OneLambda, CA) for all transplantations between 2005 and September 2008 [20]. For transplantations performed after September 2008, sera were retrospectively tested for the presence of anti-HLA antibodies with the LifeScreen Deluxe and Lifecodes single antigen beads class I and II (Immucor, GA) in case of a positive screening. Based on a comparative study between both HLA antibody assays showing roughly corresponding results at a mean fluorescence intensity (MFI) cut-off of 4000 [21], we decided to apply this cut-off. Donor-specific HLA antibodies (DSA) were assigned for HLA-A/-B/-C/-DR/-DQ by comparing bead specificities with the donor HLA type on split level.

Donor DNA isolation and genotyping

Donor DNA was extracted from peripheral blood mononuclear cells or splenocytes at the time of transplantation for HLA typing purposes by using the MagnaPure Compact system according to the manufacturer's instructions (Roche diagnostics, Basel, Switzerland) and stored at 4°C. Because all donor DNA samples were stored with a code that was not traceable to an identifiable person, permission to use the leftover DNA samples for study purposes was granted by the Biobank Research Ethics Committee of the UMC Utrecht. Genomic DNA was amplified through polymerase chain reaction (PCR) by using specific primer-pairs for the promoters of CD46, CD55 and CD59 (supplementary table 1). Following enzymatic purification, the PCR products were sequenced by using sequence-primers and fluorescent capillary electrophoresis (3730 DNA analyser, Applied Biosystems, Waltham, MA). Sequence data was analyzed with SeqScape® version 2.7 (Applied Biosystems).

Statistical analyses

All data were analyzed with SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA) and R3.2.2. Survival analyses were performed by constructing Kaplan-Meier curves and tested for significance with the log-rank test. No correction for multiple testing was performed because we carefully selected the investigated polymorphisms beforehand based on literature and frequency within the general population instead of random testing of all identified polymorphisms with the CD46, CD55 and CD59 promoter regions [22]. To adjust for potential confounders Cox multiple regression was performed. Included in the adjusted analysis were panel reactive antibodies (PRA), donor type, retransplantation and induction therapy. Results are reported as hazard ratios (HR) with 95% confidence interval and *p*-values. A *p*-value of <0.05 was considered to be statistically significant.

Results

Patient and donor characteristics

Five different polymorphisms in the promoters of CD46, CD55 and CD59 that are frequently present within the general population were sequenced. The observed genotype frequencies within our donor population are summarized in table 1 and are comparable to the frequencies that have been reported by the 1000 genomes project [23]. We will refer to the two different CD46 SNPs as A (rs2796267) and B (rs2796268).

Table 1. Overview of studied polymorphisms.

Complement protein	Polymorphism - rs number	Alleles	Genotype frequencies within cohort:
CD46	2796267 (A)	A/G	A/A: 31% G/G: 17% A/G: 52%
	2796268 (B)	A/G	A/A: 31% G/G 17% A/G: 52%
CD55	150046210 (A)	-/TAGTTACTTCCCCTCCTTCCC	+/+: 49% -/-: 43% +/-: 9%
	28371583 (B)	A/G	A/A: 54% G/G: 8% A/G: 38%
CD59	147788946	-/A	-/-: 71% A/-: 29%

Additional donor and recipient characteristics are summarized in table 2. Fifty-eight patients suffered from at least one episode of biopsy proven acute rejection within the first year.

These episodes were classified as: borderline rejection (12%), acute cellular rejection (66%), acute antibody-mediated rejection (5%) or combined antibody-mediated and cellular rejection (17%). The overall death-censored 5-year graft survival rate was 84%, 48 grafts failed during follow-up. Thirty-one patients died with a functioning graft within 5 years posttransplantation.

Table 2. Baseline characteristics.

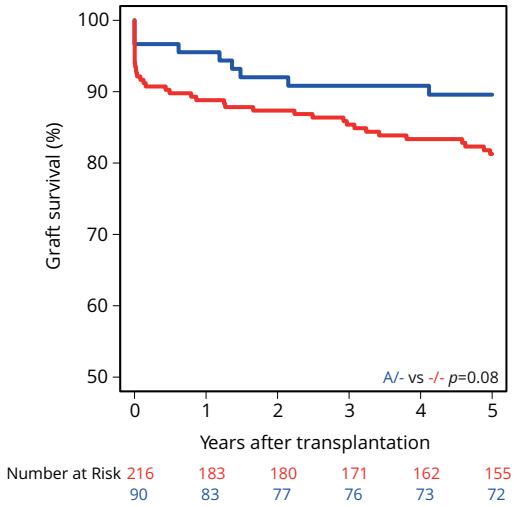
	Cohort (n=306)
Recipient age (years)	49.6 ± 13.8
Recipient sex, male	172 (56%)
Donor age (years)	51.5 ± 13.2
Donor sex, male	137 (45%)
Donor type	
• Living	136 (44%)
• Donation after brain death	85 (28%)
• Donation after circulatory death	85 (28%)
First transplant	257 (84%)
Peak panel reactive antibodies >5%	57 (19%)
Pretransplant donor-specific HLA antibodies^	33 (11%)
HLA-A, -B, -DR mismatches (no.)	2.6 ± 1.6
Cold ischemia time (hours)*	16.5 ± 6.8
Delayed graft function	77 (25%)
Induction therapy	54 (18%)

^ Data on pretransplant DSA status was missing for 5 patients. * Cold ischemia time for deceased donors only. Data are depicted as number and percentage or mean ± standard deviation.

CD46 and CD59 promoter polymorphisms are associated with acute rejection

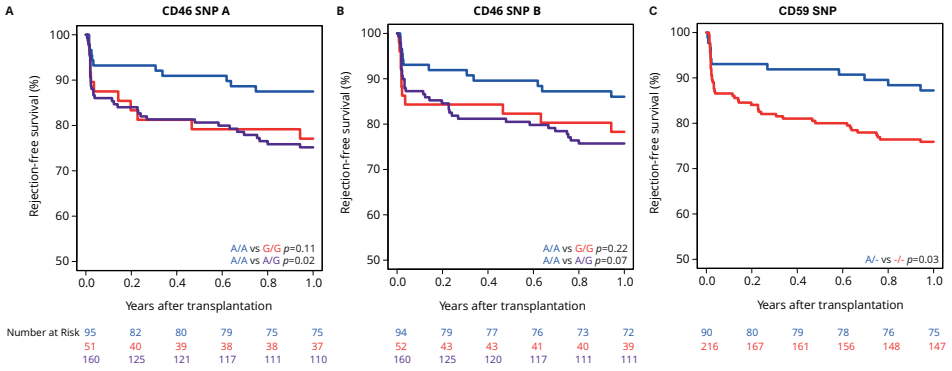
Kaplan-Meier survival analyses showed no associations between the polymorphisms in the promoters of the genes encoding for CD46 and CD55 and 5-year death censored graft survival. For the CD59 promoter polymorphism, the survival curve hints at an impaired survival of kidneys with a SNP configuration without an adenine insertion (-/-), although this is not significant (p=0.08; figure 1). Regarding freedom from acute rejection, differences were observed for the CD46 and CD59 promoter polymorphisms, but not for CD55 (data not shown). Patients receiving a kidney with at least one G allele for the CD46 SNP A showed a significantly lower freedom from acute rejection (p=0.02; figure 2A). The other CD46 SNP (B), showed a trend towards a lower freedom from acute rejection in the presence of at least one G allele (p=0.07; figure 2B). Finally, the -/- configuration of the CD59 SNP in kidney donors correlated also with an impaired rejection free survival (p=0.03; figure 2C). The observed differences for all three SNPs occur already within the first weeks post-transplantation. Types of rejection stratified for donor SNP genotype are summarized in supplementary table 2.

Figure 1. Death-censored graft survival according to donor CD59 promoter genotype.



Kidneys with the -/- SNP configuration tend to have an impaired 5-year death-censored graft survival (p=0.08).

Figure 2. Rejection-free survival according to donor CD46 and CD59 genotypes.



(A) Rejection-free survival according to CD46 SNP A (rs2796267): A/A vs. G/G (p=0.11), A/A vs. A/G (p=0.02), A/A vs. G/R (p=0.02) (B) Rejection-free survival according to CD46 SNP B (rs2796268): A/A vs. G/G (p=0.22), A/A vs. A/G (p=0.07), A/A vs. G/R (p=0.07). (C) Rejection-free survival according to CD59 SNP (rs147788946): -/- vs. A/- (p=0.03).

Recipient and transplant characteristics did not significantly differ amongst CD59 and CD46 SNP B donor genotype (supplementary table 3 and 4). There were no statistical significant differences in baseline characteristics between both CD59 donor genotypes or for CD46 SNP B. For CD46 SNP A, patients receiving a kidney with at least one G allele tended to be more often retransplant patients and subsequently also had more often

a panel reactive antibodies (PRA) above 5% and induction therapy with an IL-2 receptor antagonist (supplementary table 3). After adjustment for donor type, PRA, retransplantation and induction therapy in a Cox multiple regression model, there was still a trend towards a lower rejection-free survival (HR 1.82; 0.93-3.55) in patients receiving a kidney from a donor with at least one G allele for CD46 SNP A (table 3). The adjusted analyses, including the same covariates as for CD46 SNP A, identified CD46 SNP B and CD59 SNP as significant risk markers for acute rejection within the first year (HR 1.95 and 2.18).

Table 3. Hazard ratios for acute rejection and graft failure.

Promoter polymorphism	Rs number	Genotype	Acute rejection[±]		Graft failure[§]	
			Unadjusted	Adjusted*	Unadjusted	Adjusted*
CD46 SNP A	2796267	G/R vs A/A	2.09 (1.09-4.03)	1.87 (0.96-3.65)	1.10 (0.59-2.05)	1.03 (0.55-1.95)
CD46 SNP B	2796268	G/R vs A/A	1.80 (0.95-3.39)	1.95 (1.03-3.68)	0.88 (0.48-1.61)	0.89 (0.40-1.64)
CD59	147788946	-/- vs A/-	2.01 (1.04-3.87)	2.18 (1.12-4.24)	1.88 (0.91-3.88)	1.88 (0.90-3.89)

[±] Acute rejection within the first year.

[§] 5-year death censored graft failure.

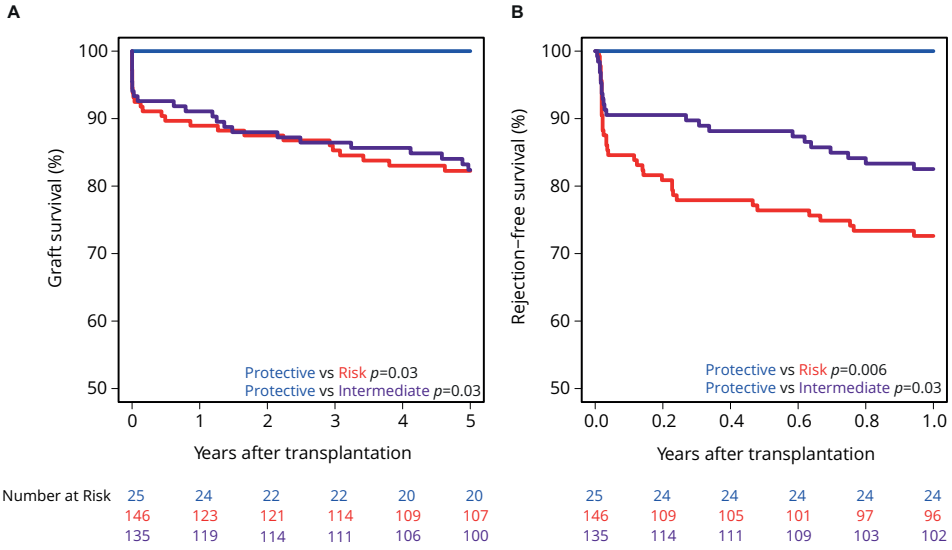
*Adjusted for panel reactive antibodies, donor type, retransplantation and induction therapy.

Data are depicted as hazard ratios with 95% confidence interval.

Because DSA are important inducers of complement activation, we also looked at the presence of pretransplant DSA in combination with donor genotypes. Five-year graft survival was the lowest in patients with pretransplant DSA receiving a kidney with a CD59 risk genotype (64%, overall p=0.02; supplementary figure 1). Moreover, rejection-free survival in patients with DSA was lower in patients receiving a kidney with a risk genotype compared with a protective genotype (p=0.03; supplementary figure 2). For CD46 SNP A and B, no differences in rejection-free survival in patients with DSA was observed between the risk and protective genotypes, though in patients without DSA rejection-free survival was lower for the risk genotypes.

Protective complotype (CD46 SNP A: A/A and CD59 SNP: A/-), risk complotype (CD46 SNP A: G/R and CD59 SNP: -/-) and intermediate complotype (CD46 SNP A: G/R and CD59 SNP: A/- or CD46 SNP A: A/A and CD59 SNP: -/-). (A) 5-year death-censored graft survival according to donor complotype: protective vs. risk complotype (p=0.03) and protective vs. intermediate complotype (p=0.03). (B) 1-year rejection free survival according to donor complotype: protective vs. risk complotype (p=0.006), protective vs. intermediate complotype (p=0.03) and intermediate vs. risk complotype (p=0.05).

Figure 3. Graft and rejection-free survival according to donor complotype.



Protective complotype yields additional preservative effects

The combined presence of multiple complement polymorphisms, a complotype, may yield additional information. Therefore, we compared patients receiving a kidney with both protective variants of CD46 SNP A and CD59 SNP alongside kidneys with both risk variants and kidneys with one protective and one risk variant, the intermediate group. Recipient and transplant characteristics of all groups are summarized in table 4, failure causes in supplementary table 4. Remarkably, none of the patients receiving a kidney from a donor with both protective genotypes suffered from acute rejection within the first year nor failed within five years (Figure 3). Kidneys with a risk or intermediate complotype showed an identical lower 5-year graft survival compared with kidneys with a protective complotype (82%, p=0.03). Regarding 1-year rejection free survival a dose-dependent effect was observed, with a lower rejection-free survival in kidneys with the risk complotype (73%) compared with kidneys with an intermediate complotype (83%, p=0.05).

Table 4. Baseline characteristics according to donor complotype.

	Protective complotype (n=25)	Risk complotype (n=146)	Intermediate risk complotype (n=135)
Recipient age (years)	50.2 ± 12.1	50.8 ± 12.0	48.3 ± 14.9
Recipient sex, male	17 (68%)	83 (57%)	72 (53%)
Donor age (years)	52.8 ± 14.8	50.6 ± 13.9	52.2 ± 12.2
Donor sex, male	11 (44%)	68 (47%)	58 (43%)
Donor type			
• Living	7 (28%)	62 (43%)	67 (50%)
• Donation after brain death	6 (24%)	44 (30%)	35 (26%)
• Donation after circulatory death	12 (48%)	40 (27%)	33 (24%)
First transplant	22 (88%)	117 (80%)	118 (87%)
Peak panel reactive antibodies >5%	3 (12%)	30 (21%)	24 (19%)
Pretransplant DSA^	4 (16%)	18 (13%)	11 (8%)
HLA-A, -B, -DR mismatches (no.)	2.7 ± 1.5	2.7 ± 1.6	2.6 ± 1.6
Cold ischemia time (hours)*	16.4 ± 9.2	17.0 ± 6.8	16.3 ± 6.2
Delayed graft function	11 (44%)	35 (24%)	31 (23%)
Induction therapy	1 (4%)	27 (19%)	26 (19%)

Baseline characteristics stratified for protective complotype (CD46 SNP A: A/A and CD59 SNP: -/-), risk complotype (CD46 SNP A: G/R and CD59 SNP: A/-) or intermediate risk complotype (CD46 SNP A: A/A and CD59 SNP: A/- or CD46 SNP A: G/R and CD59 SNP: -/-).

^ Data on pretransplant DSA status was missing for 4 patients receiving a kidney with a risk complotype and 1 patient with a donor with an intermediate complotype.

* Cold ischemia time for deceased donors only.

Discussion

This study demonstrated that two promoter polymorphisms in CD46 and one in CD59 in kidney donors correlate with a lower freedom from acute rejection within the first year posttransplantation. The survival analyses hint at a lower 5-year graft survival in patients receiving a kidney with the -/- configuration of the CD59 SNP, although this was not significant. The combined presence of both protective genotypes of CD46 SNP A and CD59 SNP has an additional preservative effect on freedom from acute rejection and 5-year graft survival. There was no association between the CD55 promoter polymorphisms and rejection-free nor graft survival.

Mechanisms by which CD46 can protect against both cellular and antibody-mediated rejection include decreased production of complement C3a and C5a and inhibition of further complement activation and amplification. Locally produced C3a and C5a can bind to antigen presenting cells (APCs) and T cells, thereby inhibiting T-cell apoptosis and enhanc-

ing T-cell proliferation and the production of co-stimulatory molecules and cytokines by APCs [4,24-27]. CD46 also acts as a direct co-stimulatory molecule for T cells, driving them towards the anti-inflammatory type 1 regulatory phenotype [28,29]. The observed early effect of both CD46 SNPs on rejection-free survival, in the absence of DSA, may be the result of enhanced T-cell activation and proliferation upon IRI induced complement activation [30].

Donor CD59 protects the allograft against rejection by hindering the formation of the membrane attack complex. Membrane attack complex formation on the donor endothelium can results in lysis, whereas sublytic levels induce endothelial cell activation and altered proliferation [31-33], augment alloreactive T-cell activation [34] and promote a prothrombotic phenotype [35-37]. Because of the limited numbers and involvement of complement in both cellular and antibody mediated rejection, we decided to pool the rejection subsets. It seems that the majority of the additional rejection episodes in the CD59 risk genotype group were classified as cellular rejection. In a substantial part of the biopsies vascular rejection was observed which is classically seen as a type of cellular rejection. However, in the presence of DSA this could also indicate antibody-mediate rejection. Indeed, for CD59 it seems like both graft failure and acute rejection within the first days posttransplantation are most prevalent in the presence of both pretransplant DSA and the donor CD59 risk genotype [38]. Pretransplant DSA have been associated with an increased risk of early failure [39]. This effect may be attributed to increased HLA antigen expression on the donor kidney within the first days due to ischemia reperfusion injury (IRI) [39]. Moreover, IRI also activates the complement cascade directly [30] and this may further contribute to the observed early effect of the complement polymorphisms.

To the best of our knowledge this is the first study that assessed the impact of promoter polymorphisms in genes encoding for complement regulatory proteins in kidney donors on transplant outcomes. Park et al. studied the CD46 SNP A in kidney transplant recipients and reported an inverse association between this polymorphism and acute and late-onset acute rejection in kidney transplant recipients [40]. However, the pathological relevance of this polymorphism in recipients in terms of acute rejection is less clear since accommodation is primarily mediated by local complement regulatory proteins expressed on the donor organ [41]. In our study, the -/- configuration of the CD59 SNP in kidney donors yielded a disadvantageous association with acute rejection. Previously, in a cohort of 137 lung transplantations, of whom 14 were diagnosed with BOS, a reverse association between the CD59 promoter polymorphism in lung donors and BOS was observed [18]. A potential explanation for the opposite effect of this SNP in kidney and lung transplantation could apply to the timing of the effect and the fact that acute and chronic rejection (including BOS) are two distinct entities [42-44]. Chronic rejection is a much more multifactorial

process requiring multiple hits, resulting in gradual parenchymal fibrosis and obliterative vasculopathy and progressive graft dysfunction [42,45], whereas acute rejection is identified by cellular and humoral attack resulting in rapid graft damage when untreated [42,46]. In our kidney transplant cohort the observed effect of the CD59 promoter SNP occurred in the first weeks posttransplantation without an effect thereafter. Therefore we hypothesize that the CD59 promoter with the -/- configuration might be more inducible through vigorous inflammation such as ischemia reperfusion injury, whereas the other CD59 variant might be more strongly transcribed in steady state. Finally, the effect of the CD59 promoter SNP may be tissue specific. It was shown that not all cell types are equally responsive to stimuli such as phorbol myristate acetate (PMA), an NF-κB activator [47], when it comes to CD59 upregulation [48]. Further studies should reveal the molecular effect of the studied CD59 promoter polymorphism.

This discovery study was set up to explore the potential associations between promoter polymorphisms in the genes encoding for complement regulatory proteins and kidney transplant outcomes. A limitation of this study is the relatively small sample size for a SNP association study and lack of replication cohort. The sample size has likely also attributed to differences in patient characteristics (retransplantation, PRA and induction therapy) when stratifying for CD46 SNP A donor genotypes, since we would not expect an association between these factors and a donor polymorphism. Retransplantation and PRA are general markers of sensitization and can include both cellular and humoral immunization. We tried to overcome this potential bias by adjusting for these factors in Cox multiple regression. Validation of our results in a larger, adequately powered, cohort is required to strengthen our findings. The incidence of rejection within the first and 5-year graft survival observed in our population are in line with numbers reported by others [2,49].Death-censored graft survival among the 51 patients transplanted without donor DNA who were excluded from further analysis, seemed to be slightly better compared with patients with available donor DNA (93%, p=0.08). However, when taking death with functioning graft into account, no significant differences in 5-year graft survival were observed between patients with available donor DNA (74%) and without donor DNA (65%, p=0.25).

It has been postulated that the combined presence of multiple polymorphisms in complement genes, a complotype, could have an even greater impact on complement activity [50-52]. For example, in age-related macular degeneration, a complement deregulation disorder, a combination of a SNP in factor H and two in factor B shows the strongest association with disease status and complement activity in vitro [51]. Therefore, we constructed a complotype combining the CD46 SNP A and CD59 SNP. The combined presence of both protective genotypes was associated with additional beneficial outcome compared with kidneys with only a single protective variant. Moreover, none of the kidneys with both

protective variants showed any signs of acute rejection within the first year nor failed during follow-up. Because of the limited number of patients receiving a kidney with a protective complotype and absence of events in this group, we could not adjust for baseline differences by Cox multiple regression. Donors with a protective complotype were more often donation after cardiac death donors and less often living donors. On the other hand, patients receiving a kidney with a protective complotype also had a lower PRA and received less often induction therapy. A potential bias in both directions can therefore not be ruled out.

In conclusion, the presented data suggests that donor polymorphisms in the promoters of CD46 and CD59 affect kidney transplant outcomes. This study opens new perspectives on the role of complement regulation in preventing acute rejection and graft failure and could add valuable information to already known risk indicators of unfavorable outcomes following kidney transplantation. We hypothesize that kidneys with a risk complotype are less capable of protecting themselves against recipient-induced complement attack. Therefore these patients may benefit from complement-targeted therapeutics like eculizumab and complement C1-inhibitor or one of the newly developed inhibitors that are currently being investigated [53]. Alongside this information could also help to determine which patients could benefit from more intensified regular immunosuppressive treatment and more frequent check ups.

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Supplementary material

Supplementary table 1. Primer sequences.

Complement protein	Primer	Direction	Sequence
CD46	PCR	Forward	5'-TGGCACTTAGGACACCCTT-3'
		Reverse	5'- CACCATGGCCGCCAGA-3'
	Sequence	Forward	5'-GCTTAATAAATTAGATTCGGAAGGG-3'
CD55	PCR	Forward	5'-ATTGTTATCCCACCCCACAC-3'
		Reverse	5'-GCACAACAGCACCAGCAG-3'
	Sequence	Reverse	5'-ACACCCTCTGGGACTCACTCT-3'
CD59	PCR	Forward	5'-TGTAAAACGACGGCCAGTGACCCAATGAGCACCTTCAAA-3'
		Reverse	5'-CAGGAAACAGCTATGACCGGCTGTGGCTGATGGCTATT-3'
	Sequence	Forward	5'-GTAAAACGACGGCCAG-3'

Primer sequences used for genomic amplification by polymerase chain reaction (PCR). PCR products were sequence in a single direction by using specific sequence primers.

Supplementary table 2. Overview of the types of acute rejection stratified for donor genotype.

	CD59		CD46 SNP A			CD46 SNP B		
	-/-	A/-	A/A	A/G	G/G	A/A	A/G	G/G
Number of patients	216	90	95	51	160	94	52	160
Rejection	48 (22%)	11 (12%)	11 (12%)	11 (22%)	37 (23%)	12 (13%)	11 (21%)	36 (23%)
Rejection type:								
Cellular	31 (14%)	8 (9%)	5 (5%)	8 (16%)	26 (16%)	8 (9%)	7 (13%)	24 (15%)
Antibody-mediated	2 (1%)	1 (1%)	1 (1%)	0	2 (1%)	1 (1%)	0	2 (1%)
Mixed	8 (4%)	2 (2%)	2 (2%)	1 (2%)	7 (4%)	1 (1%)	3 (6%)	6 (4%)
Borderline	7 (3%)	0	3 (3%)	2 (4%)	2 (1%)	2 (2%)	1 (2%)	4 (3%)

Only first rejection episodes within the first year are summarized in this table, data are depicted as number and percentage.

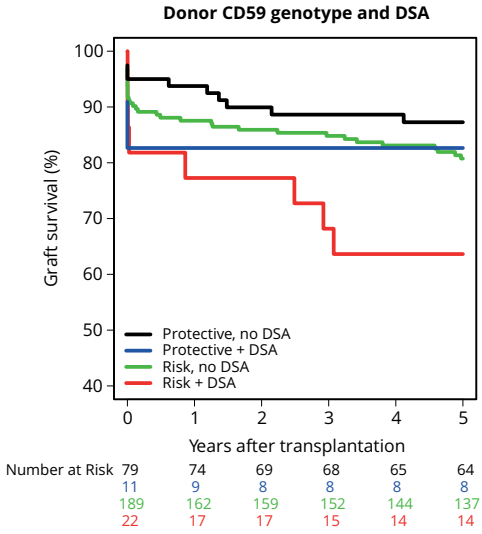
Supplementary table 3. Cohort characteristics according to donor CD59 genotype.

	Entire cohort (n=306)	CD59 -/- (n=216)	CD59 A/- (n=90)
Recipient age (years)	49.6 ± 13.8	50.3 ± 12.8	47.9 ± 14.5
Recipient sex, male	172 (56%)	120 (56%)	52 (58%)
Donor age (years)	51.5 ± 13.2	51.6 ± 13.4	51.9 ± 12.9
Donor sex, male	137 (45%)	99 (46%)	38 (42%)
Donor type			
• Living	136 (44%)	93 (43%)	43 (48%)
• Donation after brain death	85 (28%)	65 (30%)	20 (22%)
• Donation after circulatory death	85 (28%)	58 (27%)	27 (30%)
First transplant	257 (84%)	183 (85%)	74 (83%)
Peak panel reactive antibodies >5%	57 (19%)	37 (17%)	20 (22%)
Pretransplant DSA^	33 (11%)	22 (10%)	11 (12%)
HLA-A, -B, -DR mismatches (no.)	2.6 ± 1.6	2.6 ± 1.6	2.7 ± 1.6
Cold ischemia time (hours)*	16.5 ± 6.8	16.5 ± 6.7	16.5 ± 7.1
Delayed graft function	77 (25%)	52 (24%)	25 (28%)
Induction therapy	54 (18%)	35 (16%)	19 (21%)

* Cold ischemia time for deceased donors.

^ Pretransplant DSA status could not be determined for 5 patients.

Supplementary figure 1. Death-censored graft survival according to donor CD59 promoter genotype and pre-transplant DSA status.



5-year graft survival was comparable between patients with a protective (A/−) genotype kidney without DSA (90%) and with DSA (91%). For the CD59 risk genotype (−/−), graft survival was 83% in patients without DSA and 64% in patients with DSA (overall $p = 0.02$).

Supplementary table 4. Cohort characteristics according to donor CD46 genotypes.

	CD46 SNP A		
Genotype	A/A	G/G	A/G
	N=95	N=51	N=160
Recipient age (years)	49.6 ± 13.9	54.2 ± 12.0*	48.2 ± 13.2
Recipient sex, male	54 (57%)	26 (51%)	92 (58%)
Donor age (years)	52.8 ± 12.9	51.8 ± 12.0	50.6 ± 13.8
Donor sex, male	42 (44%)	22 (43%)	73 (46%)
Donor type			
• Living	38 (40%)	22 (43%)	76 (48%)
• DBD	27 (28%)	15 (29%)	43 (27%)
• DCD	30 (32%)	14 (28%)	41 (26%)
First transplant	88 (93%)	43 (84%)	126 (79%)*
Highest PRA >5%	10 (11%)	9 (18%)	38 (24%)*
Pretransplant DSA^	8 (9%)	7 (15%)	18 (12%)
HLA-A, -B, -DR mismatches (no.)	2.6 ± 1.5	2.7 ± 1.9*	2.7 ± 1.5
Cold ischemia time (hr)±	16.3 ± 6.3	16.9 ± 5.2	16.6 ± 7.7
Delayed graft function	28 (29%)	12 (24%)	37 (23%)
Induction therapy	9 (10%)	12 (26%)*	32 (20%)*

* Statistically significant compared with CD46 SNP A A/A genotype.

^ Pretransplant DSA status could not be determined for 5 patients.

± Cold ischemia time for deceased donors only.

Supplementary table 5. Failure causes according to complotype.

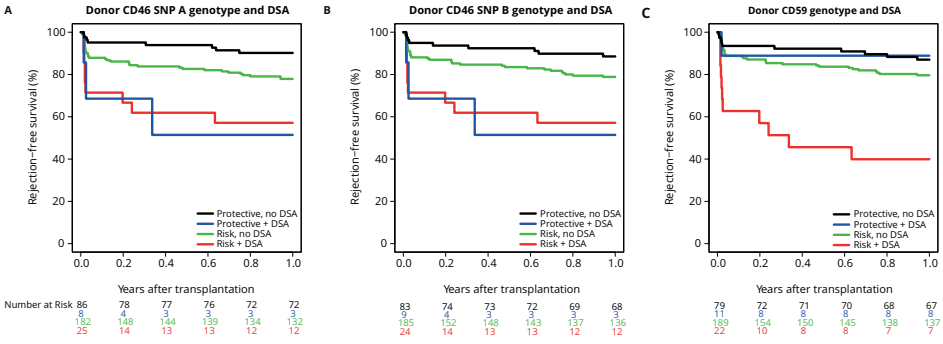
	Intermediate complotype	**Risk complotype**
	N=23	N=25
Rejection	12 (52%)	14 (56%)
Thrombosis	6 (26%)	5 (20%)
Primary non-function	3 (13%)	6 (24%)
Other	2 (9%)*	0

* Renal cell carcinoma in allograft, infection.

No failures were observed in the protective complotype group.

CD46 SNP B		
A/A	G/G	A/G
N=94	N=52	N=160
49.4 ± 13.9	52.7 ± 12.8	48.8 ± 13.2
54 (57%)	30 (58%)	88 (55%)
51.5 ± 14.5	52.6 ± 12.9	51.1 ± 12.6
41 (44%)	19 (37%)	77 (48%)
35 (37%)	24 (46%)	77 (48%)
27 (29%)	16 (31%)	42 (26%)
32 (34%)	12 (23%)	41 (26%)
83 (88%)	45 (87%)	129 (81%)
17 (18%)	10 (19%)	30 (19%)
9 (10%)	8 (16%)	16 (10%)
2.6 ± 1.5	2.7 ± 1.7	2.7 ± 1.7
15.9 ± 7.4	17.3 ± 5.3	16.7 ± 6.9
28 (30%)	13 (25%)	36 (23%)
13 (14%)	12 (23%)	29 (18%)

Supplementary figure 2. Rejection-free survival according to donor CD46 and CD59 genotypes in combination with pretransplant DSA status.



(A,B) For CD46 single-nucleotide polymorphism (SNP) A and SNP B, rejection-free survival did not differ between the protective (A/A) and risk (A/G or G/G) variant in patients with pretransplant DSA. Whereas, rejection-free survival was lower in patients without DSA receiving a kidney with a risk genotype of CD46 SNP A (p = 0.02) or CD46 SNP B (p = 0.06). (C) For CD59, rejection-free survival in patients with DSA was markedly lower for the CD59 risk genotype (−/−; p = 0.03). In patients without DSA, rejection-free survival was 87% in patients with a protective genotype (A/−) and 80% in patients with a risk genotype (p = 0.16).

Construction and primary analysis of reporter plasmids containing CD59 promoter polymorphisms

L.A. Michielsen, T. Kardol-Hoefnagel, K.A. Reedquist, M.C. Verhaar, A.D. van Zuilen, H.G. Otten.

Abstract

A single adenine insertion in the gene encoding for CD59 (rs147788946) in organ donors has been associated with rejection-free and graft survival following kidney and lung transplantation. Polymorphisms in the promoter region may affect gene transcription and expression through altered binding of transcription factors. The aim of this present study was to investigate the effect of rs147788946 on gene transcription by using reporter plasmids with the polymorphic promoter sequence. We inserted both CD59 promoter configurations in separate promoterless vectors containing the luciferase reporter gene. HeLa cells were transfected with the CD59 reporter plasmids and a reporter plasmid encoding for Renilla luciferase to correct for transfection efficiency. Six individual experiments with at least three replicates per experiment were performed. In two of these experiments HeLa cells were also stimulated with TNFα or LPS to upregulate transcription activity. Four out of six experiments, including the experiments with stimulation, indicated a significantly higher transcription activity for the CD59 promoter with adenine insertion, whereas two other experiments showed no or an opposite correlation. Potential explanations for these inconclusive results include that HeLa cells may not be suitable because of low CD59 expression compared with endothelial cells and lack of a direct effect on transcription regulation.

Introduction

The complement system is an important effector mechanism of the innate immune system [1]. In transplantation, complement is involved in ischemia reperfusion injury, rejection and recurrence of certain primary renal diseases including C3 glomerulopathy [2,3]. Complement activation is controlled by fluid-phase and membrane-bound complement-regulatory proteins [4]. CD59 is one of the membrane-bound complement regulatory proteins and functions to hinder formation of the membrane-attack complex (MAC), the terminal product of the complement cascade [4-6]. Animal studies suggest that CD59 is involved in the induction of resistance against antibody-mediated complement-mediated cell lysis, a process called accommodation [7,8].

A single adenine insertion in the promoter of the gene encoding for CD59 (rs147788946) in lung donors was found to be associated with a higher incidence of bronchiolitis obliterans, a form of chronic rejection, in lung transplant recipients [9]. Monocytes and cultured lung endothelial cells from lung donors with this insertion, showed a lower CD59 expression and these monocytes were more susceptible to complement-mediated cell lysis [9]. In kidney transplant donors, the presence of this insertion was related to an improved (early) rejection-free and graft survival [10]. This contrasting correlation could have to do with the timing of the observed effect (late in lung transplantation versus within the first few weeks in kidney transplantation) and differences in the pathogenesis of acute versus chronic rejection [10].

The promoter region is involved in transcription initiation and contributes to gene expression regulation. Polymorphisms in the promoter region may affect gene expression through altered binding of transcription factors. The adenine insertion in the CD59 promoter is located 258bp upstream of exon 1 (figure 1) and within one of the binding sites for transcription factor II D ($TF_{II}D$) [11,12]. $TF_{II}D$ is part of the RNA polymerase II preinitiation complex that is required for transcription initiation [13]. Knowledge about the downstream effect of a polymorphism is critically important for understanding of the functional role in disease and to identify novel treatment targets [14]. For sequence variations in a promoter region, a starting point may be to determine whether protein expression and gene transcription are affected [15]. The aim of this study was to investigate whether transcription activity was also lower for this CD59 promoter genotype by using reporter plasmids with the polymorphic promoter sequence. Endothelial cells are the preferred cell type for these experiments because they are directly relevant in rejection as they form the primary contact site between donor and recipient. Apart from endothelial cells that are difficult to transfect, easy to transfect HeLa and HEK293T cells are commonly used for reporter plasmid experiments [16].

Figure 1. Location of the CD59 promoter SNP (rs147788946).

(A) Location of the CD59 promoter SNP (rs147788946) within the CD59 gene on chromosome 11 according to the ensembl database [12]. (B) Close-up overview of the region containing the CD59 promoter SNP (in blue) with the binding site for transcription factor II D within the SNP region shaded in green [11,12]. Other transcription factor binding sites, not covering the CD59 promoter SNP, are not marked in this graph.

Methods

CD59 expression on different cell lines

HeLa and HEK293T cells (ATCC, Manassas, VA) were stained with anti-CD59 APC (Thermo Scientific, Waltham, MA) to determine CD59 expression with flow cytometry. HUVEC and HMEC cells (Lonza, Basel, Swiss) were stained with a mixture of anti-CD59 APC/PB/FITC against the same epitope in order to reduce the anti-CD59 APC mean fluorescence intensity (MFI) because of high expression levels on endothelial cells. All cell lines were also stained with isotype-matched control antibodies to correct for background fluorescence. Samples were measured on a BD FACS Canto II (BD Biosciences).

Genomic DNA isolation and SNP identification

DNA was extracted from peripheral blood mononuclear cells of healthy controls with the MagNA Pure Compact Nucleic Acid Isolation Kit and MagNA Pure Compact Instrument (Roche Lifesciences, Penzberg, Germany) according to manufacturer's instructions. The CD59 SNP genotype of several healthy controls was first genotyped according to a previously published protocol [10] in order to select two DNA samples: one with a homozygous adenine insertion and one without an adenine insertion at the position of the CD59 SNP. The configurations of two other SNPs in the same promoter region, that showed no correlation with transplant outcomes in lung donors, did not differ between both samples.
We amplified the 5' flanking region of the CD59 gene (-1101 to +386) with polymerase chain reaction (PCR) using the following primers: F pos -1101 (5'CAGAAGCAAGTCTCAGTCA CC-3') and R pos +386 (5'-GACGAAAGCGATGGCAG-3'). Next, restriction sites for KpnI and NcoI were introduced with a nested PCR using the following primers: F pos-1052 (5'-ggatc-cGCAAATGGCTCTAGCTGG-3') and R pos+73 (5'-CTTCTTACCaTGGCGGCG-3'). Sequence was confirmed with fluorescent capillary electrophoresis (3730 DNA analyzer, Applied Biosystems, Waltham, MA) using SeqScape® version 2.7 (Applied Biosystems).

Reporter plasmid construction

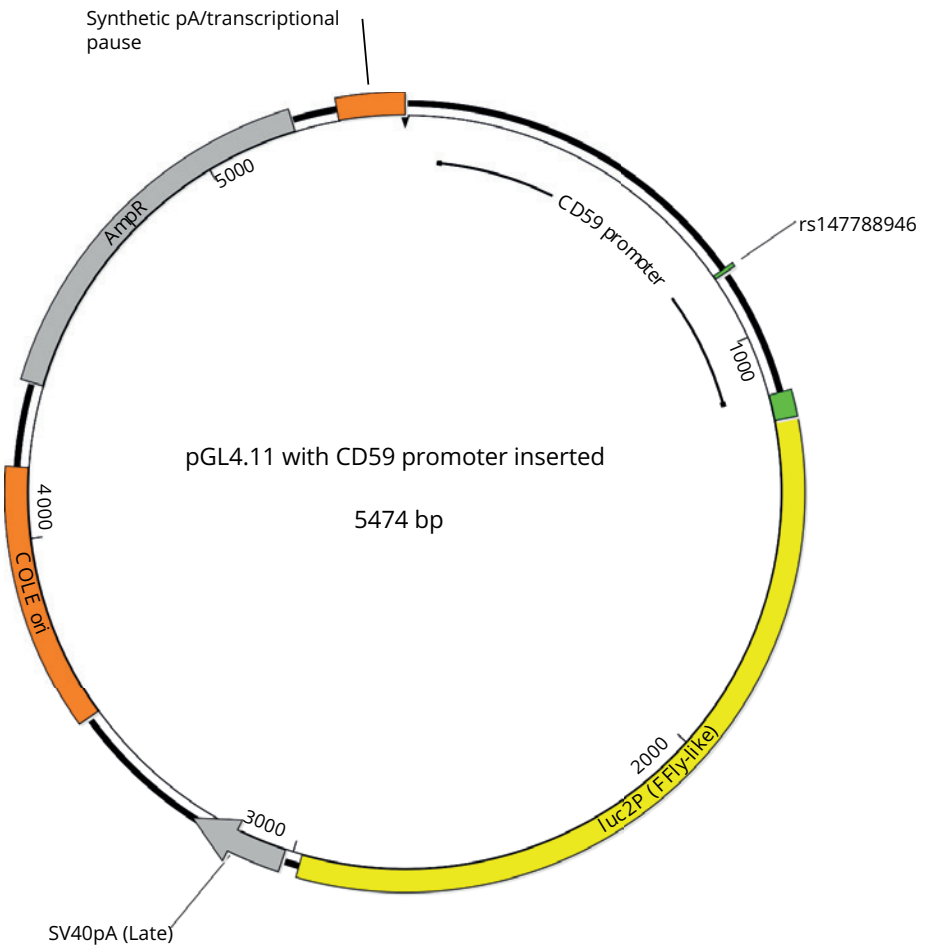
Because direct blunt-end cloning of the final PCR product into the pGL4.11 [*luc*2P] vector (Promega, Madison, WI) was not successful, we first cloned the PCR products into the blunt sides of the pJET1.2/blunt vector (Thermo Scientific) with EcoRV (New England Biolabs (NEB), Ipswich, MA). Subsequently, sticky-ends at the CD59 promoter insert were generated in two digestion steps. First, the vector with the insert was digested with EagI (NEB) and subsequently DNA polymerase and dNTPs were added to make the sticky ends blunt. Afterwards, we performed a second digestion with NcoI (NEB) to generate new sticky ends. The CD59 insert was then cloned into the sticky sides of pGL4.11 vector that were generated with EcoRV and NcoI. Sequence of the final reporter plasmid (figure 2) was confirmed with fluorescent capillary electrophoresis. Further onwards we will name the two CD59 reporter plasmids: CD59min (without adenine insertion) and CD59A (with adenine insertion).

Transfection and Luciferase assay

HeLa cells were transfected with the CD59min or CD59A reporter plasmids or the pGL4.11 [luc2P] vector with CMV promoter (positive control) or without promoter inserted (negative control). For all conditions, a co-transfection was performed with pGL4.74 [hRluc/TK] (Promega) containing the Renilla luciferase gene to correct for transfection efficiency and pMaxGFP (Lonza) to visually assess transfection efficiency. The day before transfection, cells were plated in a 24-wells plate to a density of 4x10^5 cells/well. For transfection, 100ng reporter plasmid DNA, 30ng pGL4.74 and 20ng pMaxGFP reporter plasmid DNA,

together with 300ng stuffer DNA and 0.9ul Lipofectamine 2000 (Invitrogen) was diluted in 50ul OptiMem medium per well. Following complex formation, the mixture was added to the HeLa cells and incubated for 24 hours at 37°C in a 5% CO_2/95% air. Afterwards, the media was removed and the cells were lysed with Passive Lysis Buffer (Promega). Firefly and renilla luciferase activity was measured with the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) on the Veritas™ Microplate Luminometer. The Firefly signal was normalized for the Renilla signal to correct for transfection efficiency and cell numbers. All experiments were performed at least in triplicates.

Figure 2. Reporter plasmid map with CD59 promoter insert.



Abbreviations: AmpR, synthetic β-lactamase coding region; COLE ori, ColE1-derived plasmid replication origin; luc2P gene, synthetic firefly luciferase; SV40pA, SV40 late poly(A) region.

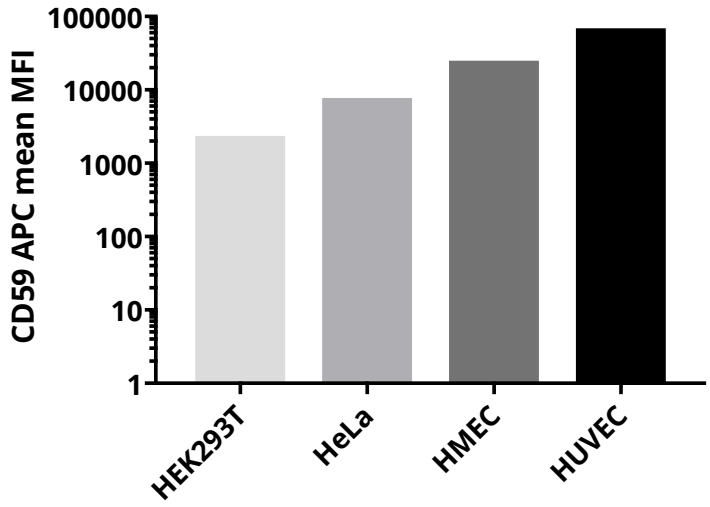
Stimulation of CD59 transcription

It was shown that CD59 transcription could be upregulated by stimulation with LPS or TNFα [17]. We hypothesized that a difference in transcription activity of the CD59 reporter plasmid might be more distinct following stimulation with these factors. Therefore, we added TNFα 10ng/ml (Miltenyi) or LPS 50ng/ml (Invivogen) 4 or 24 hours posttransfection to six wells per condition in addition to the non-stimulated conditions in two experiments. The transfected cells were then incubated for another 24 or 8 hours before lysing the cells and measuring the luciferase activity. We also checked with flow cytometry the effect of LPS or TNFα stimulation for 4, 8 or 24 hours on CD59 expression levels on HeLa cells.

Results

We first tried to set-up a protocol to transfect endothelial cells (HUVEC and HMEC) with our reporter plasmids. Despite usage of different transfection agents (lipofectamine 2000, lipofectamine 3000, lipofectamine TX, PEI and Fugene HD), transfection efficiency remained poor and the firefly and renilla luciferase signal were too low for reliable interpretation. Therefore, we looked at other cell lines that are easier to transfect. Expression analysis indicated that CD59 expression was higher on HeLa cells compared with HEK293T cells (figure 3). Therefore, we decided to continue with HeLa cells, resulting in a stable transfection protocol with an average transfection efficiency >70%.

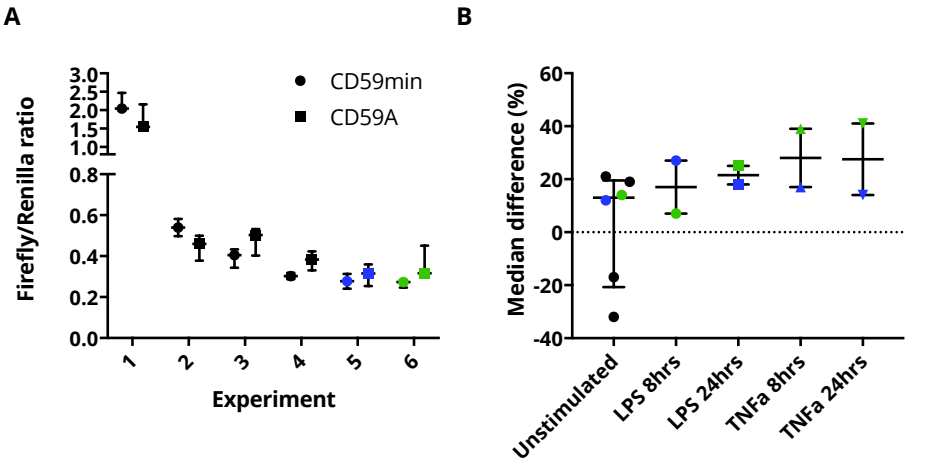
Figure 3. Constitutive CD59 expression levels on different cell lines.



Mean fluorescence intensity (MFI) of CD59 on different cell lines (n=1).

Four out of six independent luciferase experiments without stimulation indicated that the firefly/renilla ratio was significantly higher for the CD59A reporter plasmid, whereas the two other experiments indicated no significant difference or a lower ratio for the CD59A compared with the CD59min reporter plasmid (figure 4A). Pooled analysis of the median ratios of the different experiments indicated no significant difference between both reporter plasmids. In two experiments, we also stimulated HeLa cells with either LPS or TNFα for 8 or 24 hours in an attempt to increase transcription of the CD59 reporter plasmids. For each condition the transcription activity of the CD59A reporter plasmid was significantly higher compared with the CD59min reporter plasmid (figure 4B). However, the firefly/renilla ratio was not consistently increased in the stimulated conditions compared with the unstimulated conditions. In line with this, flow cytometry analysis indicated that following 8 or 24 hours of incubation with LPS or TNFα, HLA-A/-B/-C was upregulated, indicating that the HeLa cells were activated, but CD59 expression was unaffected (data not shown).

Figure 4. No consistent difference in transcription activity between CD59A and CD59min reporter plasmid.



(A) Median firefly/renilla ratio among the replicates per experiment for the unstimulated conditions. (B) Percentage of median difference in firefly/renilla ratio for the CD59A reporter plasmid conditions compared with the CD59min reporter plasmids. In two out of the six experiments, HeLa cells were also stimulated with either LPS or TNFα for 8 or 24 hours in an attempt to increase transcription activity. The symbols representing these two experiments are depicted in blue (experiment 5) and green (experiment 6) in both figures. Data represent median and interquartile range (IQR).

Discussion

The aim of this study was to assess the effect of a CD59 promoter SNP on transcription activity with a reporter plasmid assay. Six individual experiments with at least three replicates per experiment, showed no consistent difference in transcription activity between both SNP variants. In two of these experiments, we added extra conditions where HeLa cells were stimulated with LPS or TNFα following transfection in an attempt to increase transcription activity and thereby make potential differences more distinct. Transcription activity was significantly higher for the CD59A reporter plasmid for all conditions in these experiments (without stimulation and stimulated with LPS or TNFα). However, as absolute differences were small and two experiments (without stimulation) showed an opposite effect of the CD59 promoter SNP, we cannot draw any conclusions from these reporter plasmid experiments. In the next paragraphs, we will discuss the potential explanations for the inconclusive results.

We observed quite some spread in firefly/renilla ratio among replicates and experiments and in general the firefly luciferase signal was rather weak [18]. Despite several adjustments to the protocol (increased amount of reporter plasmid DNA or firefly luciferase substrate, stimulation with LPS or TNFα, altered cell numbers or longer measurement time) no major increase in the specific firefly luciferase signal was observed while transfection efficiency in general was good (>70%).

It could also be that HeLa cells are not suitable to study the effect of the CD59 promoter SNP on transcription activity. CD59 expression on endothelial cell lines was markedly higher compared with HeLa cells. This could indicate that endothelial cells may have more transcription factors involved in CD59 transcription initiation than HeLa cells. Endothelial cells are also more relevant for transplantation as the donor endothelium is the primary contact site between donor and recipient. Unfortunately, HMEC and HUVEC are notoriously troublesome to transfect [19-21] and we did not manage to set up a stable transfection protocol for either of these cell lines despite trying different transfection agents including lipofectamine 3000 and lipofectamine LTX, which are developed for hard-to-transfect cells including primary cells [21].

Another potential explanation for the inconclusive results is that the CD59 promoter SNP may have an indirect effect on transcription regulation. It has been shown for many genes that DNA loop formation between the promoter region and an enhancer is involved in regulation of the transcription initiation [22]. Because of the circular formation of the reporter plasmid (and as the reporter plasmid contains no enhancer element), we cannot rule out that the CD59 promoter SNPs influences transcription activity by interference with

loop formation. Furthermore, an individual polymorphism may have minimal functional impact itself but still affect gene expression or function by linkage disequilibrium with a set of polymorphisms [14].

Lastly, the inconclusive results may reflect that there is no distinct direct effect of the CD59 promoter SNP on transcription activity. Du et al. showed that the primary CD59 promoter region is located between -1bp to -200bp upstream of exon 1 [17] and their data suggests no additional transcription activity between -200 and -1000bp. The insertion is located -258bp upstream of exon 1 and is therefore outside the core promoter. In addition, there is a transcription factor binding site ($TF_{II}D$) within the region of the insertion, but it is unlikely that binding of this factor is affected by a single adenine insertion and moreover there are multiple other, nearby binding sites for $TF_{II}D$ [11]. Finally, for another study we repeated CD59 expression analysis on lung donor endothelial cells and also looked at CD59 expression on peripheral blood mononuclear cell subsets of lung transplant recipient both pre- and posttransplantation [23]. The only difference between both analyses was that, for the original analysis lung donor endothelial cells in culture were used, whereas in the repeated analysis we stained frozen endothelial cells straight after thawing which precludes upregulation in vitro. These repeated measurements did not confirm the previously reported expression difference on lung donor endothelial cells and monocytes between the different CD59 promoter SNP configurations (supplementary figure 1). Although expression variations amongst donor were large, the cell conditions were slightly different and we only tested a limited number of donors, this further argues the direct functional relevance of the CD59 promoter SNP.

The best way to proceed forwards may be to go first back to the clinical data. The International Genetics & Translational Research in Transplantation Network (iGeneTRAiN) contains existing genowide genotype data of over 16000 transplant patients and 11000 donors [24] and may therefore be very suitable to validate the clinical relevance of the CD59 promoter SNP in both lung and kidney transplantation. If the association with clinical outcomes can be validated, it may be worthwhile to further attempt to transfect endothelial cells with the reporter plasmid, for instance via electroporation [25].

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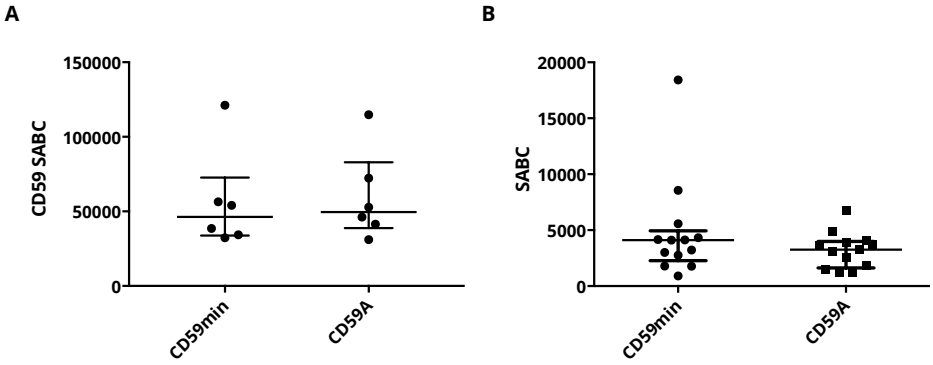
Supplementary material

Supplementary figure 1. CD59 expression according to CD59 SNP genotype.



Expression of CD59, quantified as specific antibody-binding capacity (SABC) of CD59 on lung donor endothelial cells (A) and monocytes from lung transplant recipient (B) with the CD59A or CD59min genotype of the CD59 promoter SNP.

Reduced expression of membrane complement regulatory protein CD59 on leukocytes following lung transplantation

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Front Immunol. 2018;8:2008.

Abstract

Cellular protection against undesired effects of complement activation is provided by expression of membrane-bound complement regulatory proteins including CD59. This protein prevents membrane attack complex formation and is considered to be involved in graft accommodation. Also, CD59 downregulates CD4+ and CD8+ T cell activation and proliferation. It is unknown whether CD59 expression is affected by transplantation. The aim of this study was to evaluate the quantitative CD59 antigen expression on distinct leukocyte subsets following lung transplantation (n=26) and to investigate whether this differs from pretransplantation (n=9). The results show that CD59 expression on leukocytes is significantly lower posttransplantation compared with healthy controls (p=0.002) and pretransplantation (p<0.0001). Moreover, the CD59 expression diminishes posttransplantation on all distinct lymphocyte subsets (p<0.02). This effect appeared to be specific for CD59 since the expression of other surface markers remained stable or inclined following transplantation. The highest antigen expression posttransplantation was observed on CD4+ T cells and monocytes (p≤0.002). These findings show that CD59 expression on leukocytes diminishes posttransplantation which could result in decreased resistance against complement and enhanced T cell activation. If such reduction in CD59 expression also occurs on endothelial cells from the transplanted organ this could lead to a change into a prothrombotic and proinflammatory phenotype.

Introduction

CD59 is a glycosylphosphatidylinositol-anchored complement regulatory protein that interferes with membrane attack complex (MAC) formation by blocking the binding of C9 to C5b-C8. Membrane attack complex formation may result in cell lysis, whereas sub-lytic levels can result in cellular activation, altered proliferation and the release of cyto- and chemokines through interference with certain signal transduction pathways [1-3]. For example, sub-lytic MAC levels on the endothelium can enhance alloreactive T cell activation through upregulation of noncanonical nuclear factor-kappaB (NF-κB) resulting in a proinflammatory genetic profile [4]. Furthermore, the MAC promotes a prothrombotic phenotype by inducing platelet activation and release of von Willebrand factor and procoagulent plasma membrane vesicles from endothelial cells [5-7]. Apart from preventing formation of the MAC, CD59 may directly interfere with T cell activation and proliferation upon exogenous antigen binding [8].

CD59 is widely expressed in almost all tissues and on all circulating cells, though expression levels differ greatly between cell types [9,10]. Endothelial cells exhibit much higher CD59 expression levels compared with peripheral blood mononuclear cells (PBMCs) and within PBMC subsets, including in CD4+ and CD8+ T cell subsets, differences are also observed [11,12]. However, these data are based on relative differences in mean fluorescence intensity (MFI) values whereas information on absolute CD59 antigen density on these cells is lacking.

Complement regulation is considered to play an important role in maintaining long-term allograft survival by inducing resistance against antibody-mediated complement-dependent cell lysis, a process called accommodation [13-15]. Our lab has identified a single nucleotide polymorphism (SNP) in the promoter region of CD59 (rs147788946) that is associated with lower expression levels on lung donor endothelial cells and monocytes, but not on lymphocytes, and decreased resistance against complement-mediated cell lysis. The presence of this SNP configuration in lung donors was also associated with a higher incidence of bronchiolitis obliterans syndrome (BOS) and a tendency towards impaired long-term patient survival [16]. These results support the hypothesis that CD59 expression levels on the donor endothelium correlate with graft survival. However, little is known on CD59 expression levels posttransplantation when patients are subjected to immunosuppressive therapy. Because posttransplant endothelial cells were not available since transbronchial biopsies are not routinely being performed, we used PBMCs as a model system. The aim of this study was to evaluate the quantitative CD59 antigen expression on distinct leukocyte subsets following lung transplantation and to investigate whether this differs from pretransplant CD59 antigen expression.

Patients and methods

Patients and sample collection

Twenty-nine patients who underwent lung transplantation in the UMC Utrecht between April 2004 and June 2012 were included in this study based on sample availability. From these patients, 19 patients were exclusively included in posttransplant measurements, 3 solely in pretransplant measurements and of 7 both pre- and posttransplant samples were included. In addition, nine healthy controls were also included in this study. Standard immunosuppressive therapy posttransplantation consisted of tacrolimus, mycophenolate mofetil (MMF) and prednisone with basiliximab induction therapy. Written informed consent was obtained from all patients and healthy controls. This study was approved by the Medical Research Ethics Committee of the UMC Utrecht (protocol METC 06-144) and performed in accordance with the Declaration of Helsinki.

Patient blood samples were routinely collected directly prior to transplantation and monthly during the first year posttransplantation. For this study, we used samples that were taken between month 2 and 5 posttransplantation. Because of limited cell numbers we used samples taken at different time points. PBMCs were isolated from heparin blood using Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK). Samples were frozen in RPMI medium (Thermo Fischer, Waltham, MA) supplemented with 20% fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and preserved in liquid nitrogen until analysis.

Quantification of CD59 expression

PBMCs were rapidly thawed in a warm water bath (37°C) and added to warm RPMI medium with 20% FBS, centrifuged and dissolved in phosphate-buffered saline (PBS). Cells were divided for anti-CD59 (Biolegend, San Diego, CA) staining or isotype IgG2a (Biolegend) staining in a 2:1 ratio and incubated for 30 minutes at 4°C in the dark. The median number of cells stained for CD59 was $1.7x10^6$ (IQR $1.1x10^6$; $2.2x10^6$) and for isotype IgG2a control $8.4x10^5$ (IQR $6.0x10^5$; $1.2x10^6$). Following washing, cells and QIFIKIT beads (Dako, Glostrup, Denmark) were simultaneously stained with a saturating concentration of goat anti-mouse IgG FITC in order to determine absolute CD59 expression quantified as antibody binding capacity (ABC). The QIFIKIT kit contains five bead populations with a distinct and known amount of monoclonal mouse antibody bound per microsphere bead. By constructing a calibration curve based on fluorescence intensity of the different populations plotted against their known antibody density, CD59 expression on the PBMCs can be interpolated based on their MFI. The specific antibody binding capacity (SABC) is calculated by subtracting the calculated ABC for corresponding isotype

controls from the anti-CD59 ABC. We also determined the estimated CD59 expression on lung donor endothelial cells obtained at time of transplantation (supplementary methods).

Leukocyte subsets staining and complement-mediated cell lysis

In order to study CD59 expression on different leukocyte subsets and to relate this to resistance to complement-mediated cell lysis, cells stained for CD59 or isotype control were equally divided over three different tubes designated as: (1) no HLA class I antibodies, no serum; (2) HLA class I antibodies + serum heat inactivated (HI) and (3) HLA class I antibodies + serum. Antibody-panels to identify lymphocyte subsets were added to each tube and tube 2 and 3 were concomitantly incubated with 3µl of 500µg/ml HLA class I antibody clone W6/32 (ITK diagnostics, Uithoorn, The Netherlands) for 30 minutes at 4°C in the dark. This dosage of antibodies correlated with 50% cell lysis on average in previous dose-finding experiments [16]. Pooled human serum of 2 healthy volunteers was diluted 1:5 with veronal saline buffer (Lonza, Basel, Switzerland). 25µl PBS was added to tube 1; 25 µl serum HI 1:5 was added to tube 2 and 25µl of serum 1:5 was added to tube 3. Following incubation at 37°C for 15 minutes, cells were washed with annexin-V binding buffer and incubated with annexin V PE (BD Biosciences, Franklin Lakes, NJ) and 7AAD (BD Biosciences) for 15 minutes. Samples were measured on a BD FACS LSR II with 10-color detection (BD Biosciences).

Influence of immunosuppressive drugs and immune activation on CD59 expression

To assess the effect of immunosuppressive drugs and immune activation on CD59 expression, we used whole blood and PBMCs of six healthy volunteers. Whole blood samples were spiked with: tacrolimus 10ng/ml (Selleckchem, Housten, TX), mycophenolic acid 2.5µg/ml (Sigma-Aldrich), prednisolone 21-acetate 150ng/ml (SantaCruz Biotechnology, Dalles, TX), a combination of these drugs or PBS as a negative control. Samples were incubated for 24 hours at 37°C, 5% CO2. This incubation time was selected based on preliminary time course experiments, showing a maximum effect after 24 hours of incubation. Following incubation the erythrocytes where lysed with lysing solution (BD Biosciences) and stained for flow cytometry analysis. PBMCs of the same donors were cultured for 24 hours at 37°C, 5% CO2 in the presence or absence of human T activator CD3/CD28 dynabeads (Invitrogen, Waltham, MA). Subsequently, CD59 expression on T cells was measured with flow cytometry analyses.

Antibodies

Leukocyte subsets were identified using an antibody-panel containing CD45-PO (Life Technologies) for lymphocytes, CD3-AF 700 (Biolegend) for T cells, CD14-PeCy7 (Biolegend) for monocytes, CD19-BV711 (Biolegend) for B cells and CD16/CD56-APC (eBioscience, BD Biosciences) for NK cells. T cell subsets were distinguished by CD3-AF 700 (Biolegend),

CD4-BV711 (Biolegend), CD8-PeCy7 (BD), CD27-APC-eF780 (eBioscience, San Diego, CA) and CD45RO-PB (Biolegend). Each sample was also stained with isotype-matched control monoclonal antibodies for spectral compensation and to correct for background fluorescence. Because of the extensive antibody panel, some antibodies were measured in the same fluorescence channel, e.g. CD4 and CD19, CD8 and CD14 and CD16 and CD56. A representative example of the gating strategy to identify the different lymphocyte subsets is provided in supplementary figure 1.

Statistics

All data were analyzed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). The Mann-Whitney test and Wilcoxon matched-pairs signed rank test, as appropriate, were used to compare groups. A *p*-value of <0.05 was considered to be statistically significant.

Results

Patient demographics

Patient and transplant characteristics are summarized in table 1. The percentage of patients developing BOS or acute rejection was slightly higher in the patients that were included in posttransplant analyses, though this was not significant. Nine healthy controls were also included in this study, the majority of these were woman (n=7) and their age was comparable to the lung transplant recipients (median 48 years, IQR 44; 59). The proportion of the different leukocyte subsets did not notably differ between month 2 and 5 posttransplantation (supplementary figure 2).

CD59 expression on leukocytes is markedly lower in lung transplant patients

First, we compared quantitative expression of CD59 on leukocytes of healthy controls to pre- and post lung transplantation. The results indicate that the expression of CD59 did not differ significantly between healthy controls and pretransplant samples, while it was significantly lower posttransplantation compared with pretransplantation ($p<0.0001$) and healthy controls ($p=0.002$) (Figure 1A). The number of CD59 surface molecules post lung transplantation was on average 2.9 times lower compared with pretransplantation. Next, we looked at MFI levels of different surface expression markers on leukocytes that were used to identify distinct subsets, to investigate whether this lowered expression was a general effect. Unlike for CD59 expression, CD45+ cells showed a significant increase in the combined expression of CD4/CD19, CD8/CD14 and CD16/CD56 and a tendency towards higher CD3 MFI levels following lung transplantation (Figure 1B). In comparison, the expression of CD59 on lung donor endothelial cells obtained at time of transplantation is notably higher compared with leukocytes (supplementary figure 3).

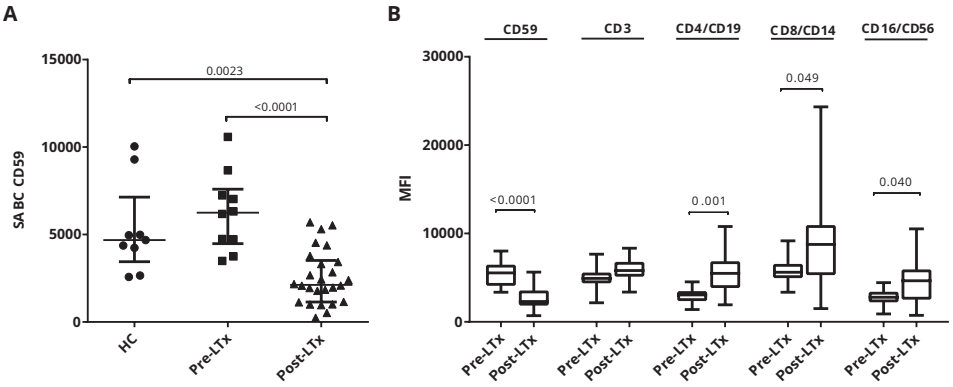
Table 1. Patient and transplant characteristics**.**

	Pretransplantation	Posttransplantation
Recipient		
Total number	10	26
Gender, male	4 (40%)	11 (42%)
Median age (years)	53 (45; 59)	50 (38; 55)
Primary disease		
• COPD	6 (60%)	11 (42%)
• Cystic fibrosis	2 (20%)	10 (38%)
• Interstitial lung disease	2 (20%)	5 (19%)
Infection		
• Cytomegalovirus (CMV) high risk	2 (20%)	5 (19%)
• Epstein-Barr virus (EBV) high risk	1 (10%)	2 (8%)
Clinical complications		
• Bronchiolitis obliterans syndrome	3 (30%)	13 (50%)
• Episode of acute rejection	1 (10%)	7 (27%)
• Patient death	4 (40%)	10 (38%)
Type of transplantation		
• Unilateral	5 (50%)	6 (23%)
• Bilateral	5 (50%)	20 (77%)
Graft ischemic time (minutes)		
• Right lung	222 (178; 265)	210 (180; 247)
• Left lung	338 (248; 395)	331 (278; 389)
Donor		
Gender, male	4 (40%)	11 (42%)
Median age (years)	49 (45; 57)	48 (39; 56)
Smoking, yes	4 (40%)	9 (35%)
Donor type		
• Donation after brain death	8 (80%)	22 (85%)
• Donation after circulatory death	2 (20%)	4 (15%)

Seven patients were included both in pre- and posttransplant analyses. EBV of CMV high risk: CMV/EBV negative recipient and CMV/EBV positive donor.

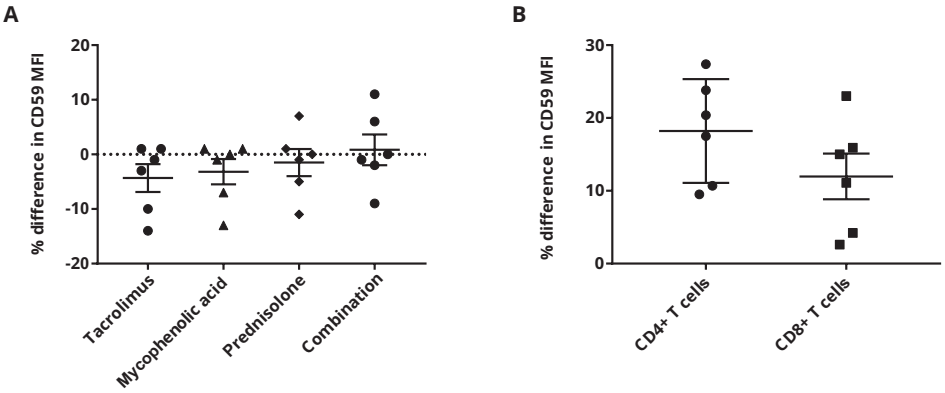
To investigate whether CD59 expression is affected by immunosuppressive therapy, we incubated whole blood samples with different immunosuppressive drugs. In this short-term experiment, incubation with immunosuppressive drugs did not result in a striking decrease in CD59 expression on lymphocytes compared with PBS (Figure 2A). Furthermore, activation of lymphocytes with CD3/CD28 dynabeads did only result in a modest increase in CD59 expression on T cells (Figure 2B).

Figure 1. CD59 expression on leukocytes of lung transplant patients is lower compared with healthy controls (HC) and patients with end-stage lung disease.



(A) Depiction of specific antibody binding capacity (SABC) of CD59 on leukocytes. Data represent median and interquartile range, symbols indicate individual values. (B) Comparison of MFI levels of different surface expression markers on CD45+ cells. Data are presented as box-and-whisker plots with boxes covering the interquartile range and median displayed within, and whiskers displaying minimum and maximum values. Data were analyzed with Mann-Whitney test.

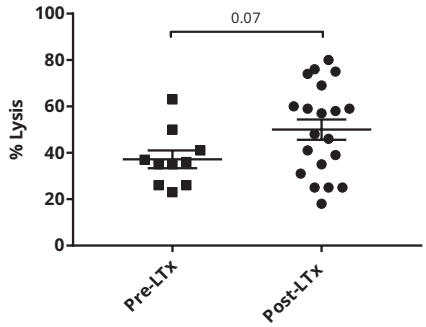
Figure 2. CD59 expression is not markedly affected by immunosuppressive drugs or immune activation.



(A) Percentage of difference in CD59 MFI on CD45+ lymphocytes for whole blood samples incubated with a single immunosuppressive drug or a combination of these tree drugs compared with PBS. (B) Percentage of difference in CD59 MFI on CD4+ and CD8+ T cells for lymphocytes stimulated with CD3/CD28 beads compared with unstimulated lymphocytes. Data represent mean and standard error of the mean, symbols indicate individual values.

In order to test whether CD59 expression levels were related to sensitivity to complement-mediated cell lysis, PBMCs were incubated with a suboptimal dose anti-HLA Class I antibody and serum as a source of complement. The mean percentage of cell lysis in samples with anti-HLA Class I antibodies and serum compared with cells that were not incubated with anti-HLA Class I antibodies was 37% for the pretransplant samples and 50% for the posttransplant samples (p=0.07; figure 3). Paired analyses, showed that the CD59 SABC was slightly (on average 7%*)* higher on the cells surviving complement-mediated cell lysis compared with living cells that were not subjected to complement-mediated cell lysis (p=0.04) and also increased compared with living cells that were subjected to anti-HLA class I antibodies and HI serum (p=0.02). Both pre- and posttransplantation there was no linear correlation between the degree of cell lysis and CD59 expression on cells surviving complement-mediated cells lysis (R=-0.48, p=0.16 and R=0.12, p=0.60).

Figure 3. Complement-mediated cell lysis pre- and posttransplantation.

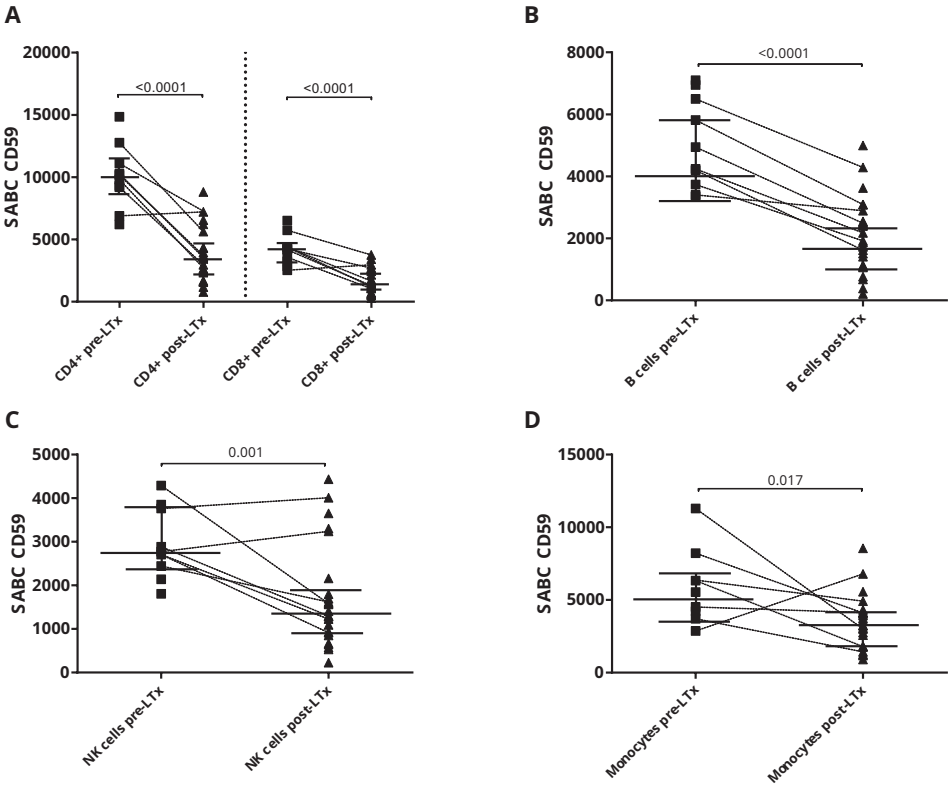


The mean percentage of cell lysis in samples with anti HLA Class I antibody and serum compared with cells that were not incubated with anti HLA Class I antibody. Data represent mean and standard error of the mean, symbols indicate individual values. Data was analyzed with the unpaired t-test.

Decreased CD59 expression following lung transplantation is observed on all lymphocyte subsets

Comparison of absolute CD59 expression on distinct lymphocyte subsets and monocytes between pre- and posttransplant samples indicated that CD59 expression decreases significantly on all subsets following lung transplantation (figure 4A-D). Paired sample analyses (n=7) showed a similar and statistical significant pattern for CD4+ and CD8+ T cells (p=0.03) and B cells (p=0.02). For NK cells and monocytes a similar decline was observed for the vast majority of patients, however this was not statistically significant (p=0.078 and p=0.11, respectively). A single patient showed increased CD59 expression levels on all subsets, except for B cells, following transplantation. For this particular patient, an infectious episode at time of posttransplant sampling could not be ruled out because of limited clinical data.

Figure 4. CD59 expression diminishes on all subsets following transplantation.

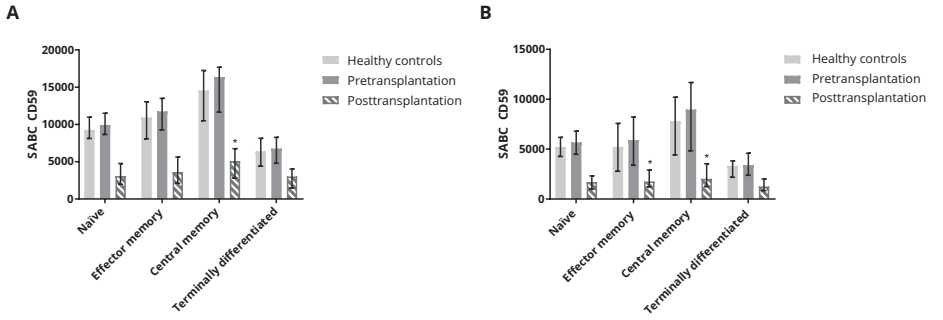


Specific antibody binding capacity (SABC) of CD59 on CD4+ and CD8+ T cells (A), B cells (B), NK cells (C) and monocytes (D) pre- and posttransplantation. A dashed line connects matched pre- and posttransplant samples. Data represent median and interquartile range, symbols indicate individual values. Data were analyzed with Mann-Whitney test.

The proportion of different subsets as part of total leukocytes did not differ significantly pre- and posttransplantation. Six out of the ten patients that were included in pretransplant measurements were on prednisone maintenance therapy prior to transplantation; CD59 expression on different leukocyte subsets did not differ between patients on steroid maintenance therapy or not (data not shown). Post lung transplantation, CD4+ T cells and monocytes showed a significantly higher CD59 expression compared with CD8+ T cells, B cells and NK cells ($p \leq 0.002$). In CD4+ T cell subsets, central memory cells showed a significantly higher CD59 expression compared with naïve ($p=0.027$) and terminally differentiated cells ($p=0.007$) (Figure 5A). For CD8+ T cells, terminally differentiated cells showed a significantly lower expression of CD59 compared with central memory ($p=0.006$) and effector memory cells ($p=0.027$) (Figure 5B). As indicated in figure 5 as a

reference, healthy controls and pretransplant samples showed comparable expression patterns amongst T cell subsets.

Figure 5. Profiling of CD59 expression on CD4+ and CD8+ T cell subsets.



CD4+ and CD8+ T cells subsets were distinguished as naïve (CD45RO-CD27+), central memory (CD45RO+CD27+), effector memory (CD45RO+CD27-) and terminally differentiated T cells (CD45RO-CD27-). (A) Posttransplantation, CD4+ central memory cells show higher expression compared with terminally differentiated (p=0.007) and naïve subsets (p=0.027). (B) In CD8+ T cells, central and effector memory cells show higher expression compared with terminally differentiated CD8+ T cells (p=0.006 and p=0.027) posttransplantation. Pretransplant samples and from healthy controls show similar expression patterns amongst subsets (not tested). Data represent median and interquartile range and were analyzed with Mann-Whitney test.

Discussion

CD59 plays a pivotal role in protecting cells from complement-mediated lysis and as such is considered to play an important role in the process of accommodation following organ transplantation [13,17,18]. Yet, little is known on the expression of CD59 posttransplantation. In this study we show that the expression of CD59 on leukocytes in lung transplant patients decreased after transplantation on all subsets. This appears not to be a general effect since the expression of other surface markers remained stable or tended to be higher following transplantation. Moreover, the expression on leukocytes of lung transplant recipients posttransplantation is also significantly lower compared with healthy controls.

We hypothesized that the observation that CD59 declines posttransplantation on all subsets may be partly attributed to immunosuppressive therapy. The expression of CD59 is mainly regulated by NF-κB and cAMP response element-binding protein (CREB) [19]. It has been reported that tacrolimus, MMF and prednisone inhibit NF-κB and CREB activity and thereby they may result in decreased CD59 transcription [20-23]. However, following

24-hours incubation of whole blood samples from healthy controls with these immunosuppressive drugs we did not observe a marked decline in CD59 expression. Nor did we observe any differences in CD59 expression levels pretransplantation between patients on prednisone maintenance therapy or not. Next, we considered that the degree of inflammation at 2 months posttransplantation and further onwards would be lower compared with pretransplantation when patients are hypoxic and suffer from recurrent infections and that this may have attributed to the declined CD59 expression. Several studies have shown that CD59 expression is increased at time of inflammation [24-26]. However, stimulation of PBMCs with CD3/CD28 dynabeads did only result in a moderate increase in CD59 expression. Therefore additional causes for the lowered CD59 expression following lung transplantation including the production of autoantibodies directed against cell-self antigens [27,28], consumption of CD59, complement-mediated lysis [29,30] and shedding should be considered [31,32].

Because of the already extensive antibody panel and limited cell numbers, we could not quantify the expression of the other membrane-bound complement-regulating proteins CD46 and CD55 in this study. Given our findings, it would of interest to investigate in future studies whether the expression levels of these latter proteins also decline following transplantation. The use of mass cytometry (CyTOF) instead of flow cytometry may overcome the issue of limited color detection possibilities. Another limitation is that we could not determine whether CD59 expression levels on endothelial cells posttransplantation also decline because transbronchial biopsies are not routinely being performed in our center. Furthermore, because of the strong expression of CD59 on endothelial cells, we can only estimate the antigen density on these large cells. Perhaps this could also be resolved by using CyTOF.

The highest expression of CD59, both pre- and posttransplantation, was observed on CD4+ T cells and monocytes. To the best of our knowledge, this is the first study to report quantitative expression levels of CD59 on different leukocyte subsets. Rao et al. previously reported that in healthy subjects the MFI was higher on myeloid cells including monocytes compared with lymphocytes [12]. Others showed that, like in our study, the relative expression is lower on NK cells compared with T cells [11] and within T lymphocytes CD45RO+ T cells show a higher expression compared with CD45RO- T cells [11,33]. CD59 serves to down-regulate CD4+ and CD8+ T cell proliferation and activation upon antigen recognition through binding of CD59 with its ligand on antigen presenting cells [8,33,34]. For monocytes, it has been postulated that they may particularly benefit from high CD59 expression because of their phagocytic function at inflammatory sites featuring vigorous complement activation [35].

Following the same lysis protocol, there was a trend towards a higher mean percentage of lysis for the posttransplant samples compared with pretransplantation. Also the expression of CD59 on cells surviving lysis was slightly higher compared with the cells that were not subjected to complement-mediated cell lysis. Though, we could not observe a linear correlation between percentage of overall cell lysis and absolute CD59 expression on leukocytes. The lowered CD59 expression on leukocytes posttransplantation may also have complement-independent effects involving altered cell signaling like enhanced T cell activation upon alloantigen presentation as mentioned above [8,33,34]. In NK cells, CD59 enhances killing via interaction with natural cytotoxicity receptors [10,36]. For monocytes and B cells no direct complement-independent roles for CD59 have been described thus far.

We hypothesize that similar mechanisms as in leukocytes may potentially also lead to lowered CD59 expression on endothelial cells within the allograft because of complement activation or shedding. Given the high expression of CD59 on lung donor endothelial cells compared with PBMCs, we hypothesize that this may not necessarily alter sensitivity to complement-mediated cell lysis but could rather favor a procoagulant and proinflammatory phenotype [4,7]. Supporting this hypothesis, we have previously reported that endothelial cells with a genotype that is associated with a lower CD59 expression secrete higher levels of fibroblast growth factor β and Interleukine-6 upon exposure to sublytic complement [16].

In summary, we show that CD59 expression on leukocytes is significantly lower in lung transplant patients compared with healthy controls and patients with end-stage lung disease. This lowered expression following lung transplantation is observed on all distinct lymphocyte subsets and monocytes. This lowered CD59 expression could be the result of complement activation or shedding of CD59. This study opens new perspective for further research to elucidate the mechanisms behind this lowered CD59 expression and to investigate whether these mechanisms also affect CD59 expression on the donor endothelium.

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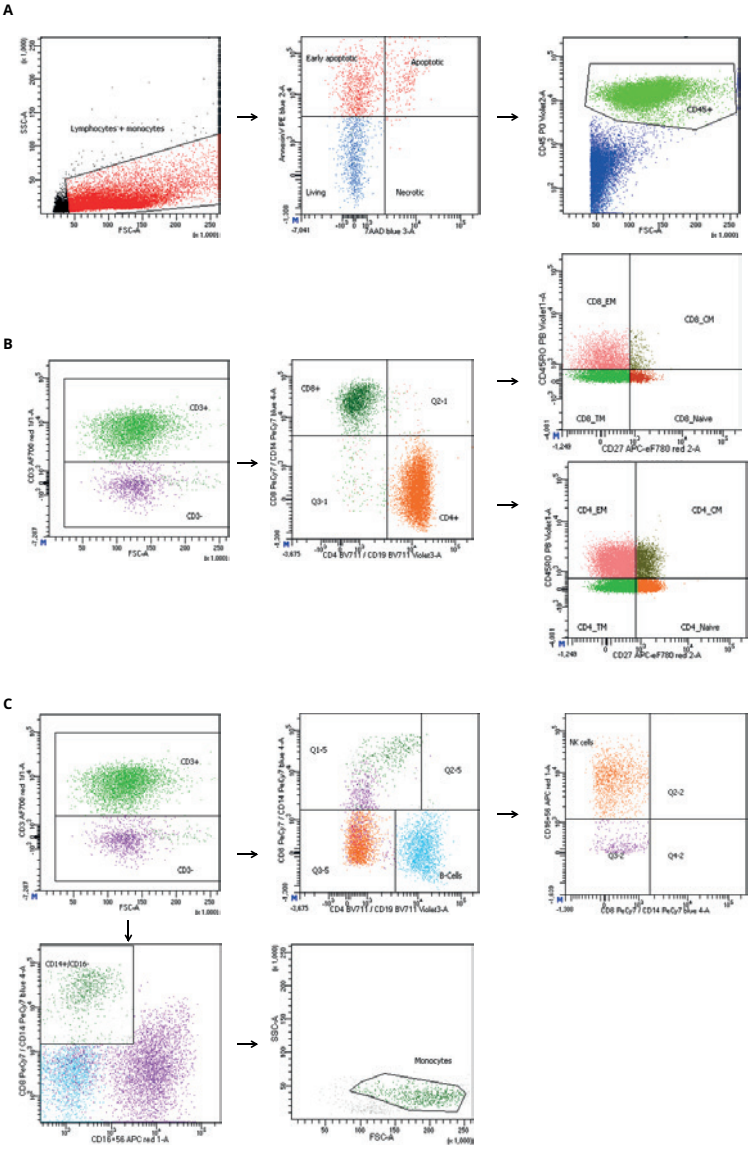
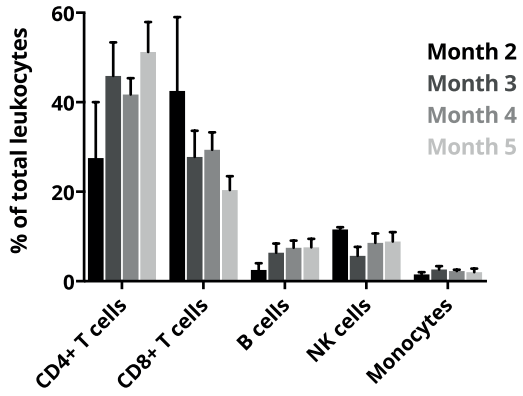
Supplementary material

Supplementary figure 1. Gating strategy.



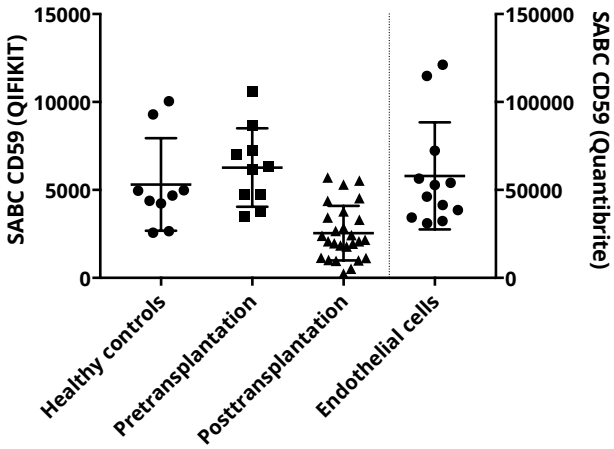
Leukocytes subsets were identified based on FSC/SSC and CD45 expression and are further characterized based on CD3. T Cells are selected from the CD45+CD3+ gate and differentiated as CD4+ and CD8+ T cells. CD4+ and CD8+ T cells subsets were distinguished as naïve (CD45RO-CD27+), central memory (CD45RO+CD27+), effector memory (CD45RO+CD27-) and terminally differentiated T cells (CD45RO-CD27-) (A). B cells are defined as CD45+CD3-CD19+ cells and NK cells as CD45+CD3-CD16+CD56+ (B). Finally classical monocytes were selected based on CD45+CD3-C14+CD16- expression and on their FSC/SSC(C).

Supplementary figure 2. Proportion of different leukocyte subsets over time posttransplantation.



Percentage of different leukocyte subsets stratified according to different sampling times posttransplantation. Data represent mean and standard error of the mean.

Supplementary figure 3. Estimated CD59 expression on endothelial cells is notably higher compared with leukocytes.



Depiction of specific antibody binding capacity (SABC) of CD59 on leukocytes calculated by using the QIFIKIT on the left y-axis and estimated SABC of CD59 on lung donor endothelial cells based on anti-CD59 PE median fluorescence intensity calculated by using Quantibrite™ beads on the right y-axis. Data represent median and interquartile range, symbols indicate individual values.

Supplementary methods

In order to relate CD59 expression on leukocytes to endothelial cells, we determined the specific antibody binding capacity (SABC) on lung donor endothelial cells obtained at time of transplantation (method for isolation has previously been described [16]). Because of the high CD59 expression on endothelial cells, cells were incubated with a mixture of anti-CD59 PE (BD Biosciences, San Diego, CA) and unlabeled anti-CD59 (Biolegend, San Diego, CA) against the same epitope. The SABC based on the anti-CD59 PE median fluorescence intensity was determined by using Quantibrite beads (BD Biosciences), containing bead populations with distinct amount of PE molecules, in order to avoid a secondary staining step with goat anti-mouse IgG FITC as used in the QIFIKIT. Since we performed competitive staining with anti-CD59 PE and unlabeled anti-CD59, the actual CD59 SABC on endothelial cells will be even higher.

Serum and urinary soluble CD59 levels in acute renal allograft rejection

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Submitted.

Abstract

Introduction

The complement regulatory protein CD59 may be shed from the cell surface upon cell activation and damage. We hypothesized that soluble CD59 (sCD59) levels may be used as a novel biomarker for acute rejection and graft function at year 1 in kidney transplant patients.

Methods

Serum and urinary sCD59 levels were measured in 10 patients with and 10 matched patients without acute rejection at time points pretransplantation, at month 1, 3, 6 and 12 and at time of a for cause biopsy. In these biopsies, CD59 expression was also quantified. In addition, we measured serum sCD59 levels at month 1 in 57 additional patients and in 10 healthy controls.

Results

Serum and urine sCD59 levels were significantly higher at time of acute rejection (p=0.001 and p=0.02). Moreover, serum sCD59 levels at month 1 posttransplantation were significantly higher in patients who developed acute rejection after month 1 but within the first year, compared with patients without rejection (p=0.002). The correlation between serum sCD59 levels at month 1 and graft function at year 1 was only limited (ρ=-0.40).

Conclusions

In conclusion, this data shows that serum and urinary sCD59 are increased in patients with acute rejection. However, it is unknown whether this increase is rejection-specific.

Introduction

Complement activation is an important effector mechanism of the innate immune system that contributes amongst others to renal allograft rejection[1]. Membrane-bound complement regulatory proteins including CD59, protect cells against complement-mediated damage [2]. CD59 (18kDa) impedes the formation of the membrane attack complex (MAC), the terminal product of the complement cascade that induces cellular activation and potentially also cell lysis [2]. Upregulation of CD59 on the donor endothelium is considered to play an important role in protection of the allograft against antibody-induced complement-mediated cell lysis [3,4]. A decreased or deficient CD59 expression has been reported in several complement-mediated diseases including age-related macular degeneration [5], paroxysmal nocturnal hemoglobinuria [6] and systemic lupus erythematosus [7].

Within the healthy kidney, CD59 is most abundantly expressed on peritubular capillaries (PTC), followed by tubular and collecting duct epithelial cells [8,9]. Very limited information on human CD59 expression at time of renal allograft rejection is available. Nishi et al. compared CD59 expression on PTCs at time of acute cellular rejection to normal human kidney tissue, classifying expression into only four grades, and did not observe any differences between both groups nor within the rejection specimens [9]. In a rat model it was shown that CD59 expression in the allograft declined with progression of acute T-cell mediated rejection [10]. For CD46 and CD55, two other membrane-bound complement regulatory proteins, expression differences on human PTCs at time of rejection have been observed, with higher expression levels being associated with a favorable graft survival or function following rejection [10,11].

Glycosylphosphatidylinisotol (GPI)-anchored proteins including CD59 can be shed from the cell surface by various processes including cellular activation and damage [12,13]. The soluble form of CD59 (sCD59), which still has complement regulatory properties, can be detected in various body fluids including urine, serum, plasma, breast milk and cerebrospinal fluid [13-16]. Urinary sCD59 usually lacks the GPI-anchor resulting in a smaller protein (average 13kDa) that can be easily filtered by the glomerulus (serum derived) or shed from tubular cells [17]. It is unknown to what extent serum and urine sCD59 levels correlate with each other.

In lung transplantation, it was observed that serum sCD59 levels are increased in patients developing bronchiolitis obliterans syndrome, even before the onset of clinical symptoms [18]. In patients with acute myocardial infarction serum sCD59 levels were also increased [13], whereas CD59 expression within the myocardium was lost [19]. Based on these observations we hypothesized that sCD59 levels are increased at time of rejection following renal transplantation and may be associated with a decrease in CD59 expression on PTC. The aim of this explorative study was to determine whether serum and urinary

sCD59 levels are increased at time of acute rejection and if serum levels at month 1 may have prognostic value in terms of the development of acute rejection and of graft function. Furthermore, we assessed whether sCD59 levels at time of rejection correlate with CD59 expression in the renal allograft.

Methods

Patients

We included patients transplanted in the University Medical Center Utrecht between May 2015 and June 2017. This explorative study consists of two parts. First, we examined whether serum and urinary sCD59 levels are increased at time of biopsy proven acute rejection (BPAR). Ten patients with BPAR within the first year and ten patients without were included in this part. Serum and urine samples were obtained at time of rejection and in addition also pretransplantation and at month 1, 3, 6 and 12 posttransplantation. Patients were matched on sex, age and donor type. In addition, paired urine and serum samples were collected from 10-healthy volunteers to identify a reference range.

In the second part, we examined to what extent serum sCD59 levels at month 1 posttransplantation relate to rejection after the first month but within year 1 and graft function at year 1. Therefore, we combined the serum sCD59 levels at month 1 posttransplantation of part 1 with measurements in 57 additional patients based on sample availability, resulting in 77 serum samples. Of these 57 additional patients, 4 experiences acute rejection within the first year.

All patients were transplanted in the University Medical Center Utrecht between May 2015 and June 2017. Biopsies were solely performed on indication (rise in serum creatinine) and evaluated by an experienced nephropathologist according to the Banff 2013 classification [20]. Clinical data was obtained from the hospital records. All patients provided written informed consent for collection of their serum, urine and clinical data in a biobank. Both the biobank and study protocol were approved by the local Biobank Research Ethics Committee (protocol 15-019 and 17-635).

sCD59 measurements

Serum and urine samples were stored at -80°C until analysis. sCD59 levels were measured in duplicate using a commercial ELISA (HK374, Hycult Biotech, Uden, The Netherlands) per manufacturers protocol. Urinary sCD59 concentrations were normalized for urinary creatinine levels that were measured with an enzymatical assay (Creatinine PAP FS, DiaSys, Holzheim, Germany) per manufacturers protocol.

CD59 staining kidney biopsy specimens

Of the patients with acute rejection in the case-control cohort, we stained one biopsy at time of acute rejection per person. Kidney biopsies were formalin-fixed and paraffin-embedded using standard procedures. Of each biopsy, 3 µm sections were cut, deparaffinised with xylene and rehydrated with a 100%, 96%, and 70% ethanol sequence. Endogenous peroxidase was blocked by H_20_2-incubation followed by antigen retrieval by boiling in citrate pH6 followed by incubation for 1h at room temperature with mouse anti-CD59 (1:200; ab9183, Abcam, Cambridge, UK). Sections were incubated with Brightvision HRP linked secondary antibody (Immunologic, Duiven, the Netherlands) and NovaRed (Vector; Torrance, CA, USA) was used as chromogen. For quantification, two trained persons blinded for sCD59 concentrations scored all biopsies independently. The intensity of the CD59 staining on the peritubular capillaries (PTC) was scored as negative (0), weak (1), moderate (2) or strong (3). This number was multiplied with the percentage of PTC staining positive for CD59 to construct an H-score ranging from 0 (negative) to 300 (maximum).

Statistics

Data was analyzed with graphpad prism 7 (Graphpad Software Inc., Sa Diego, CA). Concentrations between groups were compared with Mann-Whitney U test or unpaired t-test as appropriate. Correlations were assessed with Spearman's rho.

Results

sCD59 levels in patients with vs. without rejection

The healthy control group consisted of 4 females and 6 men and the average age was 26 years. Patient and donor characteristics of the 10 patients with and without acute rejection are summarized in table 1. The patients with biopsy proven acute rejection experienced 14 rejection episodes in total: acute borderline rejection (n=3), cellular rejection (n=3), antibody-mediated rejection (n=5) and combined cellular and antibody-mediated rejection (n=3). For two patients only serum samples were available at time of rejection and no urine samples. The median time to first rejection was 33 days (IQR 8-44).

Before looking at levels at time of rejection, we first examined serum and urinary sCD59 levels pretransplantation and at month 1, 3, 6 and 12 posttransplantation to get impression of changes over time. Serum sCD59 levels declined significantly following transplantation both for patients with rejection (median difference -18ng/ml; p=0.008) and without (median difference -21ng/ml; p=0.002, figure 1A). Urinary sCD59 levels were

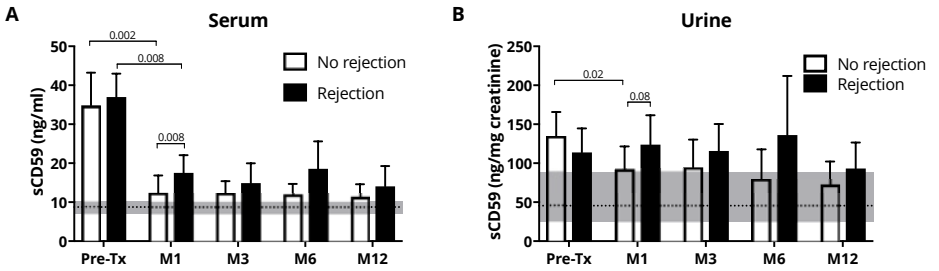
only lower in patients without rejection at month 1 posttransplantation compared with pretransplantation (median difference -49ng/mg creatinine; p=0.02, figure 1B). Serum and urinary sCD59 levels remained higher after kidney transplantation compared with healthy controls (p<0.002).

Table 1. Baseline characteristics.

	Matched group (part 1)		Entire cohort (part 2)
	No rejection (n=10)	Rejection (n=10)	(n=77)
Donor age	54.8 ± 8.5	62.6 ± 8.4	56.1 ± 9.5
Donor sex, female	4 (40%)	6 (60%)	46 (60%)
Donor type:			
• Living	5 (50%)	5 (50%)	45 (58%)
• Donation after brain death	1 (10%)	1 (10%)	12 (16%)
• Donation after circulatory death	4 (40%)	4 (40%)	20 (26%)
Recipient age	48.7 ± 8.2	48.7 ± 7.8	53.5 ± 12.4
Recipient sex, female	5 (50%)	5 (50%)	26 (34%)
Pre-emptive	4 (40%)	3 (30%)	26 (34%)
Residual urine output	9 (90%)	7 (70%)	62 (81%)
Retransplantation	3 (30%)	1 (10%)	11 (14%)
Pretransplant DSA	2 (20%)	4 (40%)	10 (13%)
Peak panel reactive antibodies	0 (0-0)	0 (0-4)	0 (0-0)
Cold ischemic period*	11.8 (11.6-13.0)	24 (22.3-24.0)	16.8 (12.5-24.0)
Delayed graft function	0 (0%)	5 (50%)	11 (14%)
Induction therapy	4 (40%)	4 (40%)	34 (44%)

* For deceased donors only.

Figure 1. sCD59 levels in serum and urine over time.

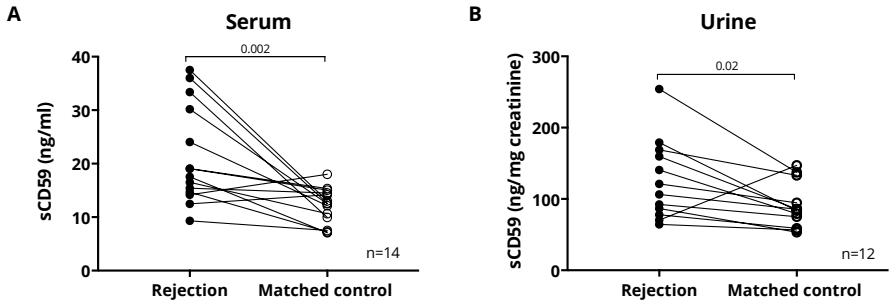


Serum (A) and urine (B) sCD59 levels over time in patients with and without acute rejection. Depicted by the grey area is the range within healthy controls. Data represent median and interquartile range (bars) and median with range (grey area). M = month; pre-Tx = pretransplantation.

Paired analyses between the matched patients with and without rejection, indicated that only serum sCD59 at month 1 posttransplantation were significantly higher in patients with rejection (median difference 4.5 ng/ml, p=0.008). In patients without rejection, serum sCD59 levels remained relatively stable over time posttransplantation, whereas quite some variation in urinary sCD59 levels over time was observed (supplementary figure 1).

Next, we compared sCD59 levels in serum and urine at time of rejection compared with the closest time matched sample of the paired recipient without rejection (figures 2A-B). sCD59 levels in serum and urine were significantly increased at time of rejection compared with their matched recipients without rejection (median difference 6.7ng/ml and 29.8ng/mg creatinine). Urine and serum sCD59 levels at time of rejection, together with rejection type and serum creatinine are provided in supplementary table 1. The overall correlation between sCD59 levels in urine and serum was moderate (ρ=0.48, p<0.0001; supplementary figure 2).

Figure 2. sCD59 levels are increased at time of acute rejection.



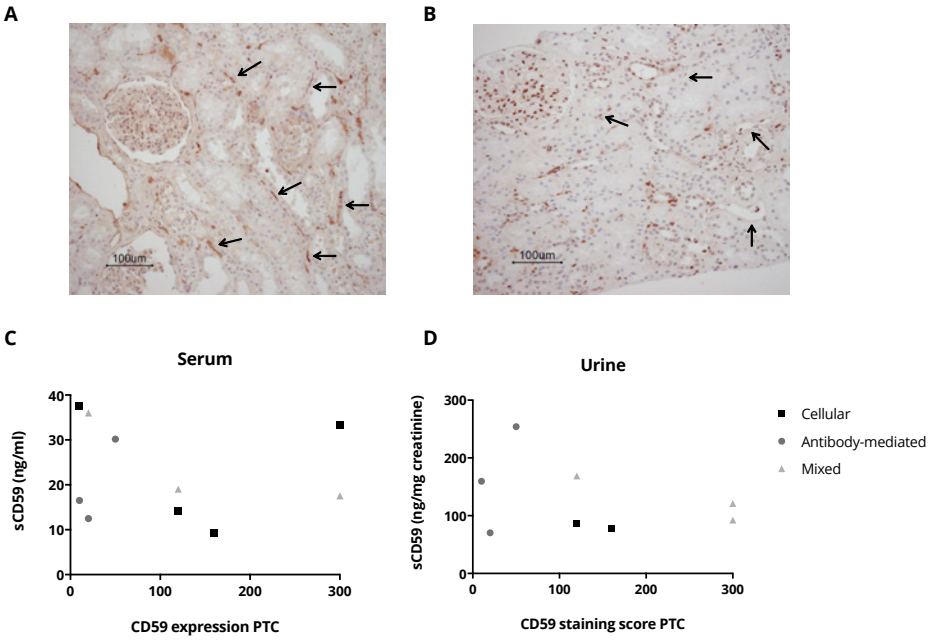
(A) Median serum concentration at time of rejection is 18.3ng/ml compared with 12.6ng/ml in controls (p=0.001). (B) In urine, the median concentration at time of rejection was 113.7ng/mg creatinine compared with 84.6ng/mg creatinine in controls (p=0.02). Data represent individual values, matched pairs are connected with a line.

To investigate whether increased sCD59 levels would correlate with CD59 expression in the allograft, we stained one kidney biopsy per patient with acute rejection for CD59. Two representative pictures of CD59 staining with a high and low intensity on PTC are provided in figures 3A-B. The correlation between the PTC CD59 expression and sCD59 levels is depicted in figures 3C-D. No distinct tubular CD59 expression could be observed in the biopsies.

Serum sCD59 levels at month 1

For the second part, we combined serum sCD59 levels at month 1 of the patients in the first part with measurements in serum of 57 additional patients. The aim was to study whether serum sCD59 levels at month 1 have prognostic value in terms of development of acute rejection and renal graft function. Within the entire group (n=77), 14 patients (18%) experienced at least one rejection episode. Six of these patients suffered from acute rejection after the first month but within the first year. Serum sCD59 levels at month 1 posttransplantation were significantly higher in this group developing acute rejection after month 1 compared with patients who did not (median difference 5.4ng/ml, p=0.002). The correlation between serum sCD59 levels at month 1 and graft function (eGFR) at year 1 posttransplantation was only limited (ρ=-0.40, p=0.002). In comparison, serum creatinine at month 1 and 1-year eGFR showed a Spearman's rank correlation coefficient of -0.72 (p<0.0001).

Figure 3. CD59 expression in renal biopsies at time of rejection.



CD59 expression in kidney biopsies with acute rejection. Arrows depict peritubular capillaries (PTC) with high expression (A, H-score 300) and low expression (B, H-score 10). The correlation between CD59 expression on PTC and sCD59 levels, classified for type of rejection, is shown in C (serum) and D (urine).

Serum sCD59 levels, independent of time of sampling, showed a positive correlation with simultaneous serum creatinine levels (ρ=0.79, p<0.0001). This association was even stronger at time of rejection (ρ=0.89, p<0.0001; supplementary figure 3A). Whereas, no significant correlation between urinary sCD59 levels, both at time of rejection or without rejection and serum creatinine was observed (supplementary figure 3B). Urinary sCD59 levels showed a limited correlation with concurrent urinary total protein levels (ρ=0.42, p=0.01).

Discussion

The results of this study indicate that sCD59 levels decline posttransplantation and are increased at time of rejection. In addition, serum sCD59 levels at month 1 are increased in patients who develop acute rejection between month 1 and 12.

Because of the size of the protein, CD59 can be easily filtered by the glomerulus as reflected by the higher concentration in urine compared with serum [21]. Given the correlation between serum sCD59 and creatinine levels, we suppose that serum sCD59 levels partly reflect renal clearance. For urine levels, no significant association with serum creatinine was observed, nor did serum and urinary sCD59 correlate very well, suggesting that at least part of the sCD59 in urine is local derived. However, also in the non-rejection group we observed quite some intra-individual variation in urine sCD59 levels over time. Although we accounted for differences in urinary flow rate by standardizing for urinary creatinine concentration [22], we cannot rule out that the usage of spot urine samples instead of 24-hours urine samples for sCD59 measurements may have attributed to this variation [23]. The exact source of urinary sCD59 remains unclear as further illustrated by contrasting results from two other studies that evaluated urinary sCD59 levels in renal conditions. Patients with membranous glomerulonephritis showed increased urinary sCD59 levels [17], whereas sCD59 was lowered in the urine of patients with diabetic nephropathy [17] and focal segmental glomerulosclerosis [24] compared with healthy controls.

Our findings are in line with observations in lung transplant recipients. Serum sCD59 levels were increased in patients with BOS, even before clinical onset of BOS [18]. In these patients, no correlation between serum sCD59 levels and serum creatinine or general markers of infection (C-reactive protein and neutrophil counts) was observed [18]. This latter suggests that sCD59 levels in these patients mirror a local inflammatory response, rather than a systemic inflammatory state. Elucidating the origin of increased sCD59 levels at time of acute kidney rejection and BOS and the exact mechanisms responsible, may also provide new insights on complement regulation in organ transplant rejection.

Because of the limited number of patients we could not make a distinction based on type of rejection. Other limitations of this study are that we do not know whether the increased sCD59 levels are rejection-specific or not and whether sCD59 levels have added value compared with serum creatinine in diagnosing acute rejection.

We stained kidney biopsy specimens of the patients with rejection, in order to test whether sCD59 levels correlated with CD59 expression. In some patients there seemed to be an inverse correlation between serum sCD59 levels and CD59 intensity in the biopsies. In addition, in the patients with solely antibody-mediated rejection, CD59 expression seemed low. Although we did not perform any statistics on this because of the limited numbers, an inverse correlation would be in line with observations in patients with acute myocardial infarction [13,19]. The patient on the upper right of figure 2C (highest CD59 expression and sCD59 serum level) suffered from delayed graft function and showed in the biopsies both signs of acute tubular necrosis and borderline rejection. Therefore, the serum sCD59 level in this patient might be disproportionally increased because of the delayed graft function given the correlation between serum sCD59 levels and serum creatinine. For the correlation between urinary sCD59 levels and CD59 expression on PTCs even less observations were present, since no urine samples at time of rejection were available in two patients. The only other study that measured both urinary sCD59 levels and glomerular CD59 expression, indicated that patients with membranous glomerulonephritis had increased urinary sCD59 levels and a decreased glomerular CD59 staining intensity compared with normal kidneys [17].

Together, this data suggests that sCD59 levels, both in serum and urine, are increased at time of rejection and serum levels at month 1 are also increased in patients who develop acute rejection afterwards. However, it is unclear whether this increase is rejection-specific or reflects injury in general. Our data on CD59 expression in kidney biopsies opens new perspectives for further studying the relevance of these expression differences and the association with sCD59 levels.

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Supplementary material

Supplementary table 1. Specification of rejection type, sCD59 and serum creatinine levels.

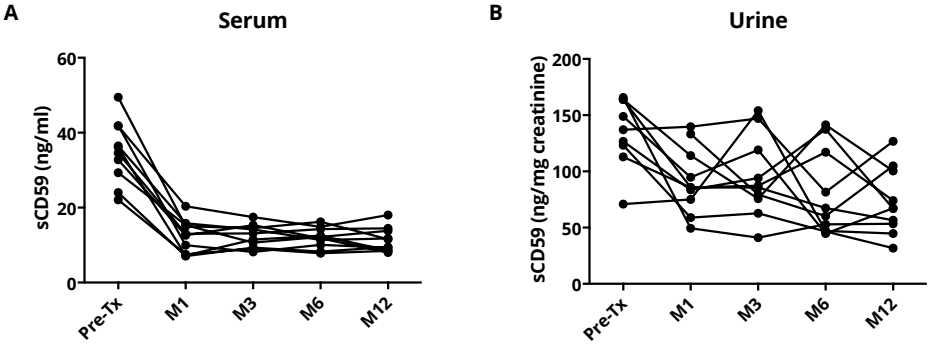
Patient	Type of rejection	Serum sCD59 (ng/ml)	Urine sCD59 (ng/mg creatinine)	Serum creatinine (µmol/L)
1	Acute borderline rejection	14.2	86.7	169
2	Acute borderline rejection*	33.4	121.1	716
3	Combined borderline cellular rejection and AMR	19.1	169.0	206
	Combined TCMR Banff IIA and AMR	15.4	140.7	185
4	TCMR Banff IIA	19.1	106.4	272
	AMR	16.5	159.6	270
	Acute borderline rejection^	24.1	64.3	399
5	AMR	12.5	70.5	248
6	TCMR IIA	36.0	-	523
7	Combined TCMR Banff IIA and AMR	9.3	77.8	97
8	AMR	17.6	92.4	206
9	TCMR IIA	37.5	-	698
10	AMR	14.7	179.2	112
	AMR	30.2	254.0	488

* Acute borderline rejection was diagnosed at time of delayed graft function.

^ Biopsy also showed 50% IFTA.

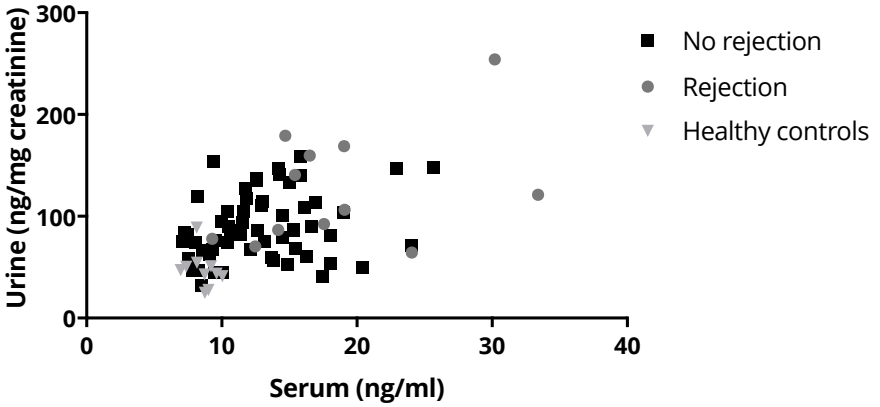
Abbreviations: AMR, antibody-mediated rejection; TCMR, T-cell mediated rejection.

Supplementary figure 1. Serum and urine sCD59 levels over time in patients without rejection.



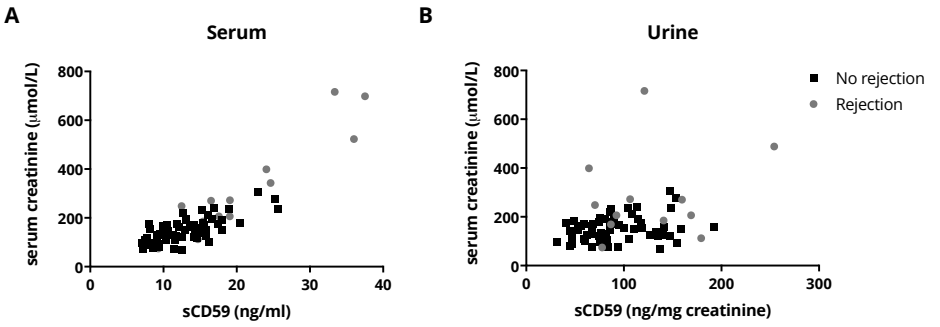
Serum (A) and urine (B) sCD59 levels over time in patients without acute rejection (n=10). Measurements within the same individual are connected by line.

Supplementary figure 2. Serum and urine levels are poorly correlated with each other.



All posttransplant measurements not taken at time of rejection where included in the no rejection group. No significant correlation between serum and urine sCD59 levels was observed for any of the groups separately or overall.

Supplementary figure 3. Correlation between posttransplant sCD59 levels and renal function.



(A) Posttransplant serum sCD59 levels at time of rejection are closely correlated with serum creatinine (ρ=0.89, p<0.0001) and to a lesser extent also in the absence of rejection (ρ=0.65, p<0.0001). (B) Urinary sCD59 levels show no significant correlation with serum creatinine.

Summary and general discussion

Summary and general discussion

Ten-year death-censored graft survival within the Eurotransplant region is around 75-80% for patients receiving a kidney from a living donor and 65-70% in patients receiving a kidney from a deceased donor [1]. The most common cause of graft failure, apart from death with a functioning graft, is antibody-mediated rejection [2-4]. A better understanding of the impact of antibodies and complement regulation on rejection and graft loss can help to identify patient groups at increased risk and guide monitoring and immunosuppressive therapy.

HLA and non-HLA antibodies in kidney transplantation

HLA antibodies

In the Netherlands, approximately 20% of the patients on the waiting list for a kidney transplant are sensitized against alloantigens, defined as a current panel reactive antibodies (PRA) percentage >5% [5]. Classical sensitizing events leading to the formation of antibodies against HLA are pregnancies, blood transfusions and previous transplantations [6]. Sensitized transplant candidates may experience prolonged waiting times [7] and if the complement-dependent cytotoxicity (CDC) crossmatch test is negative and a sensitized patient gets transplanted, there is still a greater risk of antibody-mediated injury and graft failure [7-10]. This is primarily attributed to the presence of donor-specific antibodies (DSA) that are not detected by the CDC crossmatch [10]. The luminex single antigen bead assay enables detection of these non-cytotoxic DSA and also non-donor-specific HLA antibodies (nDSA). In this thesis we studied luminex defined DSA that did not cause a positive CDC crossmatch. Multiple studies indicated that the presence of these pretransplant DSA is related to a lower graft survival on a group level [11-15], whereas the clinical relevance of nDSA remains controversial [16-19]. Possible mechanisms by which nDSA may be related with a lower graft survival include their reflection of the potential of the patient's immune system to form HLA antibodies upon allorecognition, the presence of resident donor-specific memory B-cells and epitope sharing between nDSA specificities and donor-specific antigens [18-22]. We performed a paired kidney graft analysis as part of the PROCARE consortium study, a Dutch multicenter study evaluating all transplantations between 1995 and 2005, in order to compare the clinical relevance of DSA and nDSA (**chapter 2**). A major strength of this design is that by comparing recipient pairs receiving a kidney from the same donor but with a different pretransplant HLA-antibody status, we did not have to account for differences in donor organ quality and era-dependent factors between groups. The results of this

analysis confirm that the presence of pretransplant DSA in deceased donor transplantation is related to a lower long-term graft survival compared with patients without HLA antibodies, as well as a higher incidence of acute rejection within the first year. However, the presence of nDSA in general was not related to a lower graft survival compared with patients with no HLA antibodies. Only in patients with nDSA against both HLA class I and II, who were more broadly sensitized as reflected by a markedly higher peak PRA and number of retransplants, a tendency towards a lower graft survival on the long term was observed. Because of this broader sensitization, these patients may have a greater likelihood of having resident donor reactive memory B and T cells [20,23]. Donor-specific B and T-cell ELISPOT assays could provide additional insights on whether the suggested adverse relation between nDSA class I and II is related to donor reactive memory cells [24,25]. In addition, the odds of a nDSA being donor-epitope specific despite being non-donor antigen specific might be greater in broader sensitized patients. Finally, non-HLA antibodies may also be involved in the prognosis of these patients.

Immunosuppression and pretransplant HLA antibody status

To prevent rejection, patients are treated with immunosuppressive drugs, preferably restraining the alloimmune response while limiting side effects. In order to reach this goal, an individual immunological risk-stratification needs to be made. With the introduction of luminex single antigen bead assays for (pretransplant) DSA monitoring in routine care, additional information to define the immunologic risk has become available. Given the increased incidence of acute rejection and graft loss in patients with pretransplant DSA (**chapter 2**; [11-15]), this group probably requires an intensified immunosuppressive therapy including (depletional) induction therapy. Our findings on nDSA as described in **chapter 2** could indicate that in patients with nDSA against either HLA class I or II there might be no need for intensified therapy. In patients with nDSA against both HLA class I and II, there could be a role for intensified immunosuppression, particularly in patients with a history of solid organ transplantation and a high PRA.

In **chapter 3**, we evaluated to what extent frequently used initial immunosuppressive therapies increase graft survival in immunological low-risk patients. This analysis was performed on the PROCARE cohort, including all transplantations performed in the Netherlands between 1995 and 2005. To limit the risk of confounding by indication we constructed a study population as homogenous as possible by excluding not only patients with pretransplant DSA, but also patients receiving a retransplant and/or induction therapy. We compared three regimes that were used in at least 200 patients: cyclosporine/prednisolone, cyclosporine/mycophenolate mofetil (MMF)/prednisolone and tacrolimus/MMF/prednisolone. Covariate adjusted analyses suggest that in immunological low-risk patients excellent long-term graft survival can

be achieved irrespective of the type of initial immunosuppressive therapy (cyclosporine or tacrolimus; with or without MMF), despite a higher incidence of acute rejection in the patients treated with a cyclosporine-based regime. The standard of care in immunosuppressive therapy is more and more shifting towards induction therapy in combination with tacrolimus, MMF and prednisolone maintenance therapy [26,27]. Despite the limitations of this study as described in **chapter 3**, we suggest that in selected patients at immunological low-risk but at high risk of side effects, minimization of initial immunosuppressive therapy might be a valid alternative. Elderly patients receiving a first transplant and without DSA may particularly benefit from a minimized, age-adapted strategy as they are at increased risk of infectious and cardiovascular adverse events. This minimized regime does not necessarily have to be initial double therapy, but dosage reduction or initial triple therapy followed by withdrawal of one or two drugs in the first months posttransplantation may also be valid alternatives.

Non-HLA antibodies

Patients can form antibodies against targets other than HLA. A study amongst 4000 recipients of an HLA-identical sibling transplant highlighted that PRA reflects not only reactivity against HLA-antigens but also non-HLA antigens [28]. Moreover, multiple cases of acute allograft dysfunction with histologically signs of antibody-mediated rejection (AMR) but no (detectable) circulating DSA have been described [29-31]. This phenomenon has recently been addressed in a cohort study compromising 935 transplantations [32]. In this cohort, 208 indication and per protocol biopsies met the histological criteria of AMR as defined by the Banff 2015 and 2017 working groups [33,34], but thorough HLA antibody detection revealed that 59% of these patients had no detectable DSA at time of biopsy. Graft survival in DSA negative, histological AMR cases was superior compared with patients with DSA-positive AMR suggesting that it is a distinct phenotype [32]. Potentially, non-HLA antibodies are involved in this process.

In **chapter 4**, we provide an overview of the identified targets for non-HLA antibodies in kidney transplant recipients and discuss their hypothesized mode of action and sensitizing events. These targets include both allo- and autoantigens, with most targets being ubiquitously expressed throughout the body. One of the most studied non-HLA antibody target thus far is the angiotensin II type 1 receptor (AT_1R). All studies available at time of writing chapter 4, indicated an adverse relation between AT_1R antibodies and rejection-free and/or graft survival [35-37]. Since then, a few small studies also published similar results [38-40]. However, the authors of one of the initial studies re-assessed the relation between pretransplant AT_1R antibodies and rejection-free and graft survival in a more recent (2008-2012) multicenter cohort of 940 kidney transplant recipients who

were all routinely screened for luminex defined pretransplant DSA [41]. Using the same ELISA assay and cut-off as in the original study [36], they could not confirm their previous relation between AT_1R antibodies and impaired transplant outcomes [41]. Also for other non-HLA antibodies, conflicting results have been published. Multiple explanations are possible for these contrasting observations including the lack of uniform cut-offs and potential confounding by the presence of HLA antibodies or other non-HLA antibodies. Very recently, two commercial non-HLA multiplex assays that include up to 70 non-HLA targets became available. This enables future studies to assess non-HLA antibody patterns instead of testing for a single or several non-HLA antibodies. The PROCARE consortium also developed a non-HLA multiplex assay that contains 12 different indirectly coupled non-HLA antigens [42]. Indirect coupling with a HaloTag enables full 3d-exposure of antigen for potential antibody binding.

Unlike for HLA-antibodies, there seems to be no distinct relation with classical sensitizing events and non-HLA antibody formation. Furthermore, non-HLA antibodies are known to occur in healthy individuals [43,44]. Graft injury induced by ischemia reperfusion injury, donor-specific antibody binding to the donor endothelium or chronic inflammation could result in the exposure of cryptic antigens and formation of neoantigens [45,46]. This suggests that non-HLA antibodies may arise *de novo* early posttransplantation or following acute rejection. To generate new hypothesis on sensitizing mechanisms against non-HLA antigens and to study sensitization patterns, we screened serial serum samples obtained pretransplantation and within the first year following kidney transplantation with the PROCARE non-HLA luminex assay (**chapter 5**). The results suggest that transplantation and early acute rejection, in patients under immunosuppression, do not result in direct sensitization against the investigated non-HLA antigens. It would be interesting to test whether patients do become sensitized against non-HLA antigens later on, as patients develop *de novo* DSA on median around five years posttransplantation [47]. Although our study was relatively small, it illustrates the need for further studies evaluating in depth the mechanisms leading to sensitization. The PROCARE non-HLA assay is currently also being applied to evaluate approximately 4700 pretransplant sera as part of this consortium study. This analysis may provide some clues for sensitizing events prior to transplantation. In addition, this assay together with the introduced commercial assays provides plentiful opportunities to demonstrate the clinical relevance of pre- and posttransplant non-HLA antibody patterns in established kidney transplant cohorts.

Complement regulation and accommodation

In case transplantation is performed in the presence of donor-specific antibodies, different types of immunologic outcomes are possible including rejection, accommodation and a "tolerance" state under immunosuppression [48,49]. As shown in **chapter 2**, the presence of pretransplant DSA is associated with a higher incidence of acute rejection within the first year and impaired graft survival. However, not all patients with pretransplant DSA developed acute rejection following transplantation: 1-year rejection free survival was 61% in the DSA group and 1-year death censored graft survival was even 87% in this group. Apparently DSA are not detrimental in all cases. This may have to do with antibody characteristics including epitope-specificity, subclasses and ability to fix complement [50], but also with accommodation and tolerance. Accommodation is defined as adaptation of the graft resulting in resistance against the acute pathologic effects of donor-specific antibodies and complement fixation [48,51]. Tolerance reflects changes in the immune system of the recipient leading to an environment where alloantigen recognition does not lead to an active immune response [51,52]. Clinical operational tolerance, defined as a durable state of stable and acceptable graft function without immunosuppression, is rarely obtained in kidney transplantation but partial tolerance can be induced [49,53].

Accommodation was originally described and primarily studied in AB0-incompatible organ transplantation. Biopsies from AB0-incompatible transplants frequently show C4d deposition without other signs of rejection [51]. The absence of lysis in the presence of complement fixation suggests that regulatory mechanisms are involved [51]. Endothelial cells appear to become resistant against antibody-induced complement-mediated lysis through upregulated expression of cytoprotective proteins including anti-apoptotic and complement regulatory proteins [54]. Antibody binding to A/B antigens on donor endothelial cells in vitro leads to an upregulation of CD55 and CD59 expression [55,56]. Animal studies also indicated that accommodated grafts upregulated their CD59 expression, further supporting the role of complement regulation in accommodation [57,58]. Although there are differences in AB0 an HLA antibody characteristics, and desensitization therapy in HLA incompatible transplantations appears to be less effective compared with AB0-incompatible transplantations [54], we hypothesize that some form of accommodation may occur in the setting of luminex defined DSA as well. Differences in baseline expression of complement regulatory proteins between grafts may also influence whether DSA lead to AMR or not. Very limited data on the role of complement regulation in the protection against luminex defined DSA-mediated injury exist, but it is an intriguing concept to further explore.

Genetic polymorphisms in complement proteins

Polymorphism in genes encoding for complement proteins like C3, C5 or mannose binding lectin may influence protein levels and function and thereby the immune response of the recipient. Donor polymorphisms in genes encoding for complement regulatory proteins may influence the ability of a graft to be or to become resistant against complement-mediated lysis. Genetic association studies can be conducted via genome wide association studies (GWAS) or candidate gene association studies (CGAS) [59]. GWAS studies require large populations and identified polymorphisms do not necessarily have a functional or disease relevant effect [59]. A recent GWAS study comprising an initial discovery cohort of 2094 donor-recipient pairs and a validation cohort of 5866 pairs, did not identify any correlation between donor or recipient polymorphisms and kidney graft survival [60]. As a potential explanation for the negative results, the authors note that their population was still quite small for a GWAS study and they used relatively crude outcome measures obtained from a national registry [60]. On the other side of the genetic association studies spectrum, CGAS studies are hypothesis driven and enable to study low frequency polymorphisms or smaller (hospital-based or rare diseases) study populations [59]. But also in these studies large scale validation studies are essential [61]. **Chapter 6** summarizes the literature on the relevance of complement polymorphisms in kidney transplantation. Most of the identified studies applied a CGAS approach and investigated polymorphisms in complement proteins instead of complement regulatory proteins.

In lung transplantation, a single adenine insertion in the promoter of the gene encoding for CD59 of lung donors was associated with bronchiolitis obliterans syndrome (BOS), a form of chronic lung allograft dysfunction [62]. Based on this observation, we hypothesized that donor polymorphisms in the promoter region of complement regulatory proteins may also be associated with acute rejection or graft survival in kidney transplantation. Therefore, we evaluated two polymorphisms in the promoters of CD46 and CD55 and the adenine insertion in the CD59 promoter in 306 kidney donors (**chapter 7**). Patients receiving a kidney from a donor without an adenine insertion in the CD59 promoter showed a lower 1-year rejection free survival and a tendency towards a lower 5-year death censored graft survival. When we combined CD59 donor status with pretransplant DSA status, we observed that the combination of DSA and CD59 risk genotype was particularly associated with an unfavorable prognosis. For CD46, one of the SNPs (CD46 SNP A) also showed a significant relation and the other SNP (CD46 SNP B) showed a borderline significant relation with 1-year rejection-free survival. It has been shown in age-related macular degeneration, a complement deregulation disorder, that the combined presence of multiple polymorphisms in complement genes, a complotype, has a greater impact on complement activity than a single polymorphism

[63,64]. Therefore, we constructed a complotype combing CD46 SNP A an CD59 SNP. Although numbers were low, none of the patients receiving a kidney form a donor with both protective SNP genotypes experienced rejection within the first year nor failed with five years.

Previous functional assays showed that the risk haplotype of both CD46 promoter polymorphisms is associated with a lower transcriptional activity compared with the protective haplotype [65]. Moreover, CD46 SNP B is located within a binding site of the transcription factor CBF-1/RBP-Jk and potentially disrupts binding [65]. The effect of the CD59 promoter polymorphism on transcription activity is unknown. We attempted to study this by constructing reporter plasmids with the polymorphic promoter sequence (**chapter 8**). However, six individual experiments in HeLa cells with at least three replicates per experiment showed inconsistent results. Potentially, HeLa cells are not suitable to study to study the effect of the CD59 promoter polymorphism, as the expression on HeLa cells is much lower than on endothelial cells. Unfortunately, we did not manage to set-up a stable transfection protocol for endothelial cell lines. One of the other explanations for the inconclusive results is that there is no distinct direct effect of the CD59 promoter SNP on transcription activity. The insertion is located outside of the core promoter (-258bp upstream of exon 1) as luciferase experiments indicated to additional transcriptional activity between -200 and -1000bp [66].

This uncertainty of whether there is a functional effect of the CD59 promoter polymorphism further stresses the importance of validating our findings. Nevertheless, the complotype seems a promising concept and it would be interesting to eventually construct a complotype that does not only include donor polymorphisms but also recipient polymorphisms. In patients with age related macular degeneration, a combination of two polymorphisms in factor B and one in Factor H showed the highest association with disease status and complement activity in plasma in vitro [64]. It would be interesting to test this complotype in kidney transplant recipients and eventually combine this with our donor complotype. In addition to complement polymorphisms, functional polymorphisms in Fcγ-receptors influence the effectiveness of monocytes to react to IgG bound to target cells [67,68]. As such these polymorphisms may affect the recipient's immune response upon DSA binding to the donor endothelium [69,70].

Changes in expression CD59 posttransplantation

In addition to hindering formation of the membrane attack complex, CD59 also has complement-independent properties including direct interference with T-cell activation and proliferation upon exogenous antigen binding [71,72]. As little is known on the CD59 expression posttransplantation, we compared CD59 expression on leukocyte subsets

posttransplantation with pretransplant and healthy control expression levels (**chapter 9**). Unlike the other studies performed in this thesis, this study was performed in lung transplant recipients. The results of this study indicate that CD59 expression decreased posttransplantation on all leukocyte subsets and is also significantly lower compared with healthy controls. This lowered expression may lead to increased sensitivity to complement-mediated cell lysis as reflected by a trend towards a higher percentage of leukocyte lysis following in vitro incubation with a suboptimal dose anti-HLA class I antibodies and serum posttransplantation. In case of sublytic C5b-C9 complex formation, a lowered CD59 expression may lead to increased endothelial cell activation, resulting in heightened production of inflammatory chemo- and cytokines [73]. The exact mechanisms responsible for this lowered expression are unknown but could include consumption of CD59 and shedding. As transbronchial biopsies posttransplantation are not routinely being performed, we could not assess whether such a reduction in CD59 expression also occurs on the donor endothelium posttransplantation. But we hypothesize that similar mechanism may also affect CD59 expression on the donor endothelium. Given the presumed protective properties of CD59 including the role in accommodation, it would be worthwhile to perform further research into the mechanisms attributing to CD59 expression regulation posttransplantation.

It is uncertain whether the findings described in chapter 9 also apply to kidney transplant recipients and the allograft. The inflammatory and innate immune responses following transplantation are not identical between lung and kidney transplantation [74-77], as reflected by the markedly higher rejection rates in lung transplantation compared with kidney transplantation [78,79]. This may have to do with exposure of the graft to environmental antigens in lung transplantation [79], differences in the strength of the immune response to the donor organ [75] and on average a higher number of HLA mismatches in lung transplantation because HLA matching is not included in the (Eurotransplant) lung allocation algorithm [5].

Soluble CD59 in kidney transplantation

Current monitoring of graft function and rejection is primarily based on serum creatinine and proteinuria. These markers are unspecific for rejection and therefore numerous urinary and serum biomarkers have been evaluated as potential additional biomarker [80,81]. Among the most promising novel biomarkers for rejection are urinary chemokine C-X-C motif ligand (CXCL)9 and CXCL10 [82-84], with the first randomized clinical trials evaluating these biomarkers being performed at the moment (Clinicaltrials.gov: NCT03140514, NCT03206801, NCT03465397). In **chapter 10**, we carried out a pilot study to evaluate whether serum and urinary soluble CD59 (sCD59) levels have potential to be used as a novel biomarker for acute rejection and graft function at year 1. The rationale behind this was that GPI-anchored proteins like CD59 can be shed from the membrane upon cellular

activation and injury [85,86] and thereby sCD59 may be increased during acute kidney injury. The results indicated that serum and urinary sCD59 levels were increased at time of acute rejection and serum sCD59 levels at month 1 posttransplantation were significantly higher in patients who developed acute rejection after month 1 but within the first year. However, the correlation between serum sCD59 levels and graft function at year one was only limited and serum sCD59 and serum creatinine levels were closely correlated. Based on this study we cannot conclude whether the increase in sCD59 is rejection specific or reflects injury in general and whether sCD59 levels have added value over serum creatinine as a biomarker. In order to assess this more frequent serial samples are necessary and also in patients with other causes of acute allograft dysfunction.

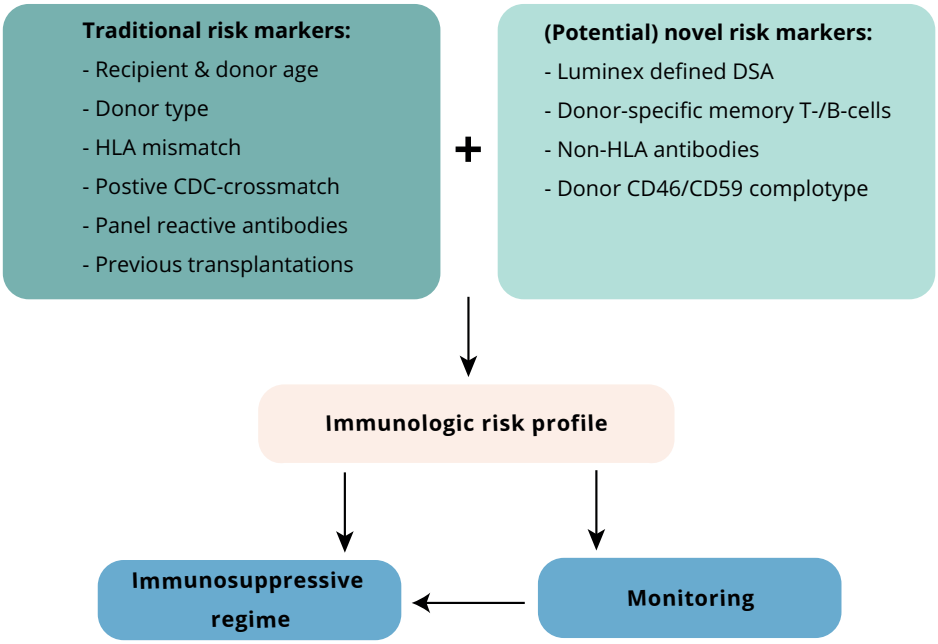
In **chapter 10** we quantified the CD59 expression in biopsy specimens taken at time of acute rejection. Although numbers were too low to perform statistics, CD59 expression seemed low in patients with pure antibody-mediated rejection and in some patients an inverse correlation between CD59 intensity and serum sCD59 levels was observed. This preliminary data opens further perspectives on the relevance of these expression differences and the potential association with sCD59 levels. For CD46 and CD55, it has been shown that staining intensity at time of acute rejection is related to improved graft function and survival following rejection [87,88]. It would be interesting to expand the number of biopsies in order to relate expression levels to outcome following rejection. Moreover, comparison of CD59 expression levels at time of (antibody-mediated) rejection to (matched) 0-hour biopsies and protocol biopsies from patients with pretransplant DSA but without signs of acute rejection might provide clues on whether accommodation also occurs in patients with pretransplant DSA.

Future perspectives

It is well established that DSA form a barrier to establishing a long-term functioning kidney graft. Yet, conformity on how to apply this information in personalized immunosuppressive protocols is lacking because there are no uniform criteria to define DSA and to ascribe a level of risk to a DSA [89]. In an effort to resolve this unmet need the first "Sensitization in Transplantation: Assessment of Risk" working group was organized in 2017 [89]. A critical literature review by a multidisciplinary team resulted amongst others in recommendations for HLA typing and HLA antibody diagnostics and assessment of a patient's capacity for alloimmune memory [89]. These recommendations will likely improve alloimmune risk assessment and hopefully will be adopted in clinical trials. However, as illustrated by the findings of this thesis, a complete immunologic risk assessment is far more complex and includes both recipient and donor factors.

With the horizon of new immunosuppressive drugs to replace current standard of care being unclear, there is a distinct need for a transition from empiric to precision medicine [90,91]. Integration of the pretransplant antibody profile, donor (and recipient) complotype, with other risk markers including donor-specific memory B or T-cell cells, HLA mismatches and recipient age may result in a more complete immunologic risk profile [90,92]. This risk profile can then be used for personalized immunosuppressive strategies but also posttransplant surveillance to assess immunosuppression adequacy (figure 1).

Figure 1. Pretransplant immunologic risk assessment.



In low-risk patients no induction therapy, a minimized maintenance regime and monitoring based on serum creatinine and tacrolimus trough level monitoring may suffice. For high-risk patients desensitization and/or depletional induction therapy in combination with triple maintenance therapy and surveillance monitoring including protocol biopsies, DSA and urinary biomarker screening may be necessary [90]. Hopefully, this approach with a tailor made immunosuppressive therapy and follow-up scheme will eventually reduce the incidence of AMR and improve graft survival.

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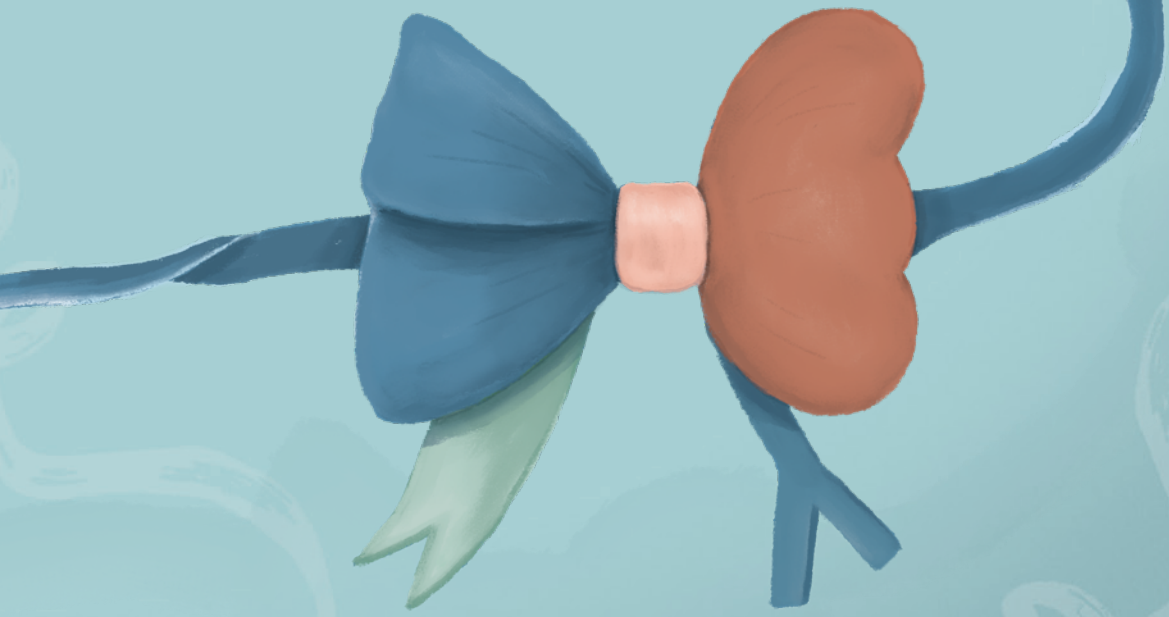
Appendices

Nederlandse samenvatting
Dankwoord
List of publications
Curriculum Vitae



Nederlandse samenvatting

Nederlandse samenvatting

Patiënten met eindstadium nierfalen hebben een niertransplantatie nodig om niet afhankelijk van dialyse te zijn. De belangrijkste oorzaak van transplantaatfalen is afstoting. Bij afstoting wordt de donornier door het afweersysteem van de patiënt herkend als lichaamsvreemd waarna een afweerreactie tegen de nier op gang wordt gebracht. Dit kan zowel vroeg na transplantatie optreden als jaren later. Acute afstoting is meestal goed te behandelen met extra afweeronderdrukkende medicijnen, terwijl chronische afstoting vaak moeizaam te stoppen is en op den duur veelal tot transplaatverlies leidt. Een van de oorzaken van afstoting is de aanwezigheid van antilichamen gericht tegen de donornier. Op de donornier zitten, net als op de meeste van onze eigen cellen, verschillende herkenningspunten (antigenen) waaronder HLA. Het is genetisch bepaald welke HLA antigenen op de cellen voorkomen. Tenzij een donornier van een eeneiige tweeling afkomstig is, is de kans aanmerkelijk dat er verschillen in HLA antigenen tussen de donor en ontvanger zitten. De ontvanger kan antilichamen vormen tegen deze niet-eigen HLA antigenen van de donor, dit noemen we donor-specifieke HLA antilichamen (DSA). DSA kunnen zowel al voor de transplantatie aanwezig zijn door eerdere transplantaties, zwangerschappen of bloedtransfusies, maar ook nieuw na transplantatie ontstaan.

Met een kruisproef kan worden getest of een ontvanger DSA heeft. Hierbij worden witte bloedcellen van de donor (met daarop HLA antigenen van de donor) aan bloed van de ontvanger toegevoegd. Als een deel van de witte bloedcellen hierop dood gaan, is er sprake van een positieve kruisproef en dit is in principe een contra-indicatie voor niertransplantatie omdat de kans op directe afstoting groot is. Met nieuwere technieken waaronder de Luminex kunnen we echter ook DSA aantonen die niet krachtig genoeg zijn om een positieve kruisproef te veroorzaken, maar die wel het risico op afstoting en transplantaatfalen verhogen. Daarnaast kan de Luminex techniek ook HLA antilichamen die niet gericht tegen de donor zijn (niet-donor specifieke HLA antilichamen) identificeren, de relevantie van deze antilichamen is echter nog niet uitgekristalliseerd.

Hoewel luminex-gedefinieerde DSA het risico op afstoting en transplantaatverlies verhogen, heeft meer dan de helft van de patiënten met Luminex-gedefinieerde DSA nog steeds een functionerende transplantatienier na 10 jaar. Één van de verklaringen hiervoor is dat sommige donornieren mogelijk beter in staat zijn om zich zelf te beschermen tegen antilichaam-geïnduceerde schade dan anderen. Zulke schade kan onder andere tot afstoting leiden door activatie van het complement systeem. De donornier heeft complement-regulerende eiwitten die beschermen tegen complement activatie.

Het primaire doel van dit proefschrift was om de impact van antistoffen en complement regulatie op transplantatie uitkomsten te bestuderen. Een beter begrip hiervan kan helpen om betere matches tussen ontvangers en donoren te vinden en om gerichter in te grijpen in patiënten met een groot risico op afstoting om zo de transplantaatoverleving te verbeteren.

Antilichamen

In **hoofdstuk 2** hebben we ontvangers van een nier van dezelfde overleden donor maar met een verschillende pretransplantatie HLA-antilichaam status vergeleken. De resultaten bevestigden dat de aanwezigheid van pretransplantatie DSA geassocieerd is met afstoting en verminderde transplantaatoverleving. Terwijl patiënten met niet-donor specifieke HLA antilchamen in zijn algemeen geen slechtere prognose hadden ten opzichte van de gepaarde patiënten zonder HLA antilichamen.

De aan- of afwezigheid van DSA kan gevolgen hebben voor de keuze en intensiteit van afweeronderdrukkende medicijnen (immunosuppressiva). Zo hebben patiënten met DSA mogelijk extra medicijnen en/of hogere doseringen nodig. Omdat de luminex techniek pas sinds een aantal jaren standaard wordt gebruikt, is de informatie over de aan/afwezigheid van DSA in veel studies die immunosuppressiva hebben vergeleken niet meegenomen. Hierdoor zijn patiënten met DSA mogelijk ten onrechte als laag-risico patiënten geclassificeerd. In **hoofdstuk 3** beschrijven we de resultaten van een studie waarin we drie verschillende behandelingen hebben vergeleken in bijna 1700 laag risico transplantaties (eerste transplantatie en geen DSA). De resultaten tonen dat met alle drie de behandelingen een goede transplantaatoverleving kan worden bereikt ondanks verschillen in voorkomen van afstoting.

Naast HLA antigenen, komen er ook andere antigenen op onze cellen voor (non-HLA antigenen). Ook hier kunnen patiënten antilichamen tegen vormen (non-HLA antilichamen). In **hoofdstuk 4** geven we een overzicht van de geïdentificeerde non-HLA antilichamen bij niertransplantatiepatiënten en de veronderstelde mechanismen waardoor deze antilichamen schade aan de transplantatienier kunnen veroorzaken. Het lijkt erop dat deze antilichamen via andere mechanismen worden gevormd dan HLA-antilichamen. Schade aan de donornier kan leiden tot blootstelling aan verborgen eiwitten of veranderingen van eiwitten, wat weer zou kunnen leiden tot antilichaamvorming tegen deze eiwitten. Op basis hiervan denken wij dat patiënten na de transplantatieprocedure of na afstoting mogelijk nieuwe non-HLA antilichamen vormen. Om dit te onderzoeken, hebben wij in bloed van transplantatiepatiënten voor en na transplantatie gekeken naar de reactiviteit tegen 12

verschillende non-HLA antigenen (**hoofdstuk 5**). De resultaten laten zien dat transplantatie en afstoting binnen het eerste jaar bij patiënten die afweeronderdrukkende medicijnen gebruiken, niet direct tot antilichaamvorming tegen de onderzochte antigenen leiden.

Complement regulatie

Het complement systeem is een van de verdedigingsmechanismen van het lichaam tegen niet eigen materiaal zoals bacteriën, maar ook een donornier. Sommige HLA- en non-HLA antilichamen zijn na binding aan de donornier in staat om het complement systeem te activeren en kunnen op die manier afstoting veroorzaken. De meeste cellen hebben echter ook eiwitten die complement activatie remmen. CD46, CD55 en CD59 zijn complement-regulerende (-remmende) eiwitten die onder andere op de donornier zitten. Mogelijk beïnvloedt de hoeveelheid (expressie) complement-regulerende eiwitten op een orgaan de kans op antilichaam-gemedieerde afstoting.

Genetische variaties (polymorfismes) kunnen de hoeveelheid of functionaliteit van eiwitten, waaronder complement (regulerende) eiwitten, beïnvloeden. **Hoofdstuk 6** geeft een overzicht van de onderzochte genetische variaties in complement (regulerende) eiwitten bij niertransplantatie. Bij longdonoren is een polymorfisme in het CD59 gen geassocieerd met het optreden van chronische afstoting bij patiënten na longtransplantatie. Om dit bij niertransplantatie te onderzoeken hebben we bij nierdonoren naar 2 polymorfismes in CD46 en CD55 en één in CD59 gekeken (**hoofdstuk 7**). Een polymorfisme in CD46 (CD46A) en in CD59 waren beiden gerelateerd met afstoting in het eerste jaar na transplantatie. Geen van de patiënten die een nier kregen met zowel de gunstige CD46A als CD59 variant, kregen namelijk afstoting binnen het eerste jaar noch verloren hun nier binnen vijf jaar na transplantatie. Voor het tweede onderzochte polymorfisme in CD46 (CD46B) zagen we eveneens een neiging naar meer afstoting binnen het eerste jaar, terwijl de polymorfismes in CD55 niet gerelateerd waren met afstoting of transplantaatoverleving.

Polymorfismes hebben niet altijd een direct (functioneel) effect op hoeveelheid of functionaliteit van eiwitten. In tegenstelling tot voor de onderzochte CD46 polymorfismes, is nog onduidelijk of het CD59 polymorfisme ook een functioneel effect heeft. Om dit te onderzoeken hebben wij reporter plasmiden met de verschillende CD59 varianten gemaakt (**hoofdstuk 8**). Deze plasmiden bevatten naast een deel van het CD59 gen een tweede gen voor een lichtgevend eiwit. Door de hoeveelheid geproduceerd licht te meten, kun je activiteit van het CD59 gen bepalen. Herhaalde experimenten lieten echter geen eenduidig verschil in activiteit tussen beide CD59 variaties zien. Mogelijk heeft het onderzochte polymorfisme in CD59 dus geen direct effect op de transcriptie en daarmee expressie van het eiwit.

CD59 is niet alleen een complement-regulerend eiwit, maar heeft ook andere beschermende effecten zoals remming van T-cel activatie en proliferatie na binding aan lichaamsvreemde antigenen. Er is echter maar weinig bekend over de expressie van CD59 na transplantatie. Om dit te onderzoeken hebben wij de expressie op witte bloedcellen na longtransplantatie vergeleken met witte bloedcellen voor transplantatie en van gezonde vrijwilligers (**hoofdstuk 9**). De resultaten laten zien dat de hoeveelheid CD59 op witte bloedcellen na transplantatie lager is ten opzichte van voor transplantatie en bij gezonde vrijwilligers. Deze lagere CD59 expressie leidt mogelijk tot grotere gevoeligheid voor complement-gemedieerde schade en als een dergelijke daling ook in het donororgaan optreedt, zou dat ongunstig voor de prognose kunnen zijn.

Monitoring van de functie van de transplantatienier gebeurt momenteel vooral door naar de nierfunctie (het kreatinine) en eiwit in de urine te kijken. Deze markers zijn echter heel aspecifiek en niet alleen verhoogd bij afstoting; om afstoting te kunnen herkennen is het daarom noodzakelijk om een nierbiopt te verrichten. In **hoofdstuk 10**, hebben wij een pilot-onderzoek gedaan om te kijken of vrij circulerend CD59 in urine of bloed potentie heeft om afstoting of transplantaatfunctie te voorspellen. Eiwitten zoals CD59 kunnen bij celschade of activatie namelijk van de cel worden afgeknipt en op die manier vrij circulerend in bloed of urine worden gevonden. Ten tijde van acute afstoting bleken de circulerende CD59 levels in bloed en urine inderdaad verhoogd en de hoeveelheid CD59 in het bloed op maand 1 was gemiddeld genomen hoger bij patiënten die tussen maand 1 en jaar 1 afstoting ontwikkelden ten opzichte van patiënten zonder afstoting. Echter, de CD59 waarden in bloed waren sterk gecorreleerd met de nierfunctie en derhalve kunnen wij niet concluderen dat het meten van CD59 in bloed (of urine) van toegevoegde waarde is ten opzichte van het vervolgen van de nierfunctie.

Slotopmerkingen

Om patiënten de beste behandeling te kunnen geven, is het belangrijk om een goede inschatting te kunnen maken van het risico op afstoting (immunologisch risico). Deze inschatting omvat, zoals geïllustreerd door de bevindingen uit dit proefschrift, niet alleen de aan-/afwezigheid van DSA, maar veel meer ontvanger- en donorkenmerken zoals non-HLA antilichamen en complement polymorfismen. Integratie van al deze kenmerken leidt waarschijnlijk tot een nauwkeurige inschatting en dit kan vervolgens gebruikt worden bij het voorschrijven van afweeronderdrukkende medicijnen en de controle van patiënten na transplantatie. Patiënten met een laag risico op afstoting hebben mogelijk een minder intensieve afweeronderdrukkende behandeling en minder frequente en uitgebreide controles nodig ten opzichte van patiënten met een hoog risico.

Dankwoord

Dankwoord

Na ruim vier jaar hard werken is mijn proefschrift af. Dit had ik nooit kunnen doen zonder de hulp en betrokkenheid van vele anderen. Om te beginnen wil ik alle transplantatie-patiënten, donoren en hun families bedanken. Zonder hen had dit proefschrift niet tot stand kunnen komen.

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List of publications

List of publications

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Curriculum vitae

Curriculum vitae

Laura Alexandra Michielsen was born in Tiel on April 17th 1989. After completing secondary school at the "Veluws College" in Apeldoorn, she started studying Medicine at Utrecht University in 2007. During her study, Laura spent two months at Hue Central Hospital, Vietnam, for a clinical elective in Infectious Diseases. She received her medical degree in 2013 and started working as ANIOS Internal Medicine in the St. Antonius Hospital in Utrecht. In 2014, Laura was given the opportunity to start her PhD research project at the departments of Nephrology and Hypertension and Laboratory of Translational Immunology at the University Medical Center Utrecht. The research of this thesis was supervised by Prof. dr. M.C. Verhaar, dr. A.D. van Zuilen and dr. H.G. Otten. From October 2018, Laura is working as ANIOS Internal Medicine in the St. Antonius Hospital in Nieuwegein. In January 2019 she will start her residency in Internal Medicine under the supervision of dr. P. de Jong and Prof. dr. H.A.H. Kaasjager.